P-NITROPHENOL UDP-GLUCURONYLTRANSFERASE GENE EXPRESSION IN 3-METHYLCHOLANTHRENE AND BUTYLATED HYDROXYANISOLE INDUCED RAT LIVER

A DISSERTATION

SUBMITTED TO THE FACULTY OF ATLANTA UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

GODWIN AJUZIEOGU ANANABA

DEPARTMENT OF BIOLOGY

ATLANTA, GEORGIA

MAY, 1988

ABSTRACT

BIOLOGY

ANANABA, GODWIN A.

B.S., North Carolina State University at Raleigh, 1980
M.S., North Carolina State University at Raleigh, 1982

P-NITROPHENOL UDP-GLUCURONYLTRANSFERASE GENE EXPRESSION IN 3-METHYLCHOLANTHRENE AND BUTYLATED HYDROXYANISOLE INDUCED RAT LIVER

Advisor: Dr. Juarine Stewart

Dissertation dated May, 1988

One of the fundamental problems in eukaryotic gene expression is to understand the effects of multiple induction. Here, the effect of single and combined treatment of rats with 3-MC and BHA on p-nitrophenol UDPGT gene expression was investigated. Antibody against the purified 3-MC form of UDPGT (M_r 57K) cross-reacted and differentially absorbed out activity from all the other treatments. Monospecific antisera preferentially recognized the 3-MC and BHA + 3-MC forms of the enzyme. Tryptic digest analysis showed conserved and variable amino acid residues between BHA and 3-MC UDPGT. Moreover, additive accumulation of polysomes, total RNA and specific poly(A^+) RNA was demonstrated. Lambda gtll rat liver cDNA library was immunoscreened. Based on these data, we contend that BHA and 3-MC induce different forms of UDPGT and as such modulate gene expression by activating two or more separate genes.

ACKNOWLEDGEMENTS

First, I am grateful to God who gave me the patience and determination to accomplish this milestone in my academic career. I cannot thank my major advisor, Dr. Juarine Stewart enough for giving me the opportunity to work in her laboratory. She provided me the most conducive environment to do my work. I am thankful to my lab mates: Carlton Boston, JoAnn McCrary and GayLeslie Brown. Also, I thank Paul Scott, Edwina Jones and Angela Reid who in one way or another contributed in this research. I am indebted to all members of my committee: Drs. John Browne, Errol Archibold, Arthur Williams and Lycurgus Muldrow who assisted at all phases of this research. I thank the Atlanta University, Biology Department family: faculty, staff and students very much for their motivating comments. Most of all, I have to thank my family: my wife, Gloria Nnenne, my son, Nkwachi Noble and my daugther, Ogechi Nancy for their endurance, encouragements and inspiration. Thank you to the entire family of late Chief John. W. J. Ananaba, "Ezeji No. 1 of Ngwaland," especially my mother, Madam Eleanor J. Ananaba who laid the foundation and made sacrifices for my education. It is unfortunate that my brother Solomon Onyenweuwa did not live to see this work completed. "May his soul rest in peace." To the Nigerian Federal Government and all my friends, I am very grateful for all they did to assist me all these years.

TABLE OF CONTENTS

LIST OF FIGURES

PAGE

LIST OF TABLES

LIST OF ABBREVIATIONS

A_{260}, A_{280}	Absorbance at wavelength of 260 and 280 nanometer
Am	Milliampere
AMV	Avian myeloblastosis virus
BHA	Butylated hydroxyanisole
BRL	Bethesda Research Laboratories
BSA	Bovine serum albumin
cAMP	Cyclic adenosine 3'5'-monophosphate
cDNA	Complementary Deoxyribonucleic acid
CFU	Colony forming unit
Ci	Currie
COOH	Carboxyl functional group
CPM	Counts per minute
dATP	Deoxyadenosine triphosphate
d(C)	Deoxycytidine
dCTP	Deoxycytidine triphosphate
DEAE	Diethyl amino ethyl
dGTP	Deoxyguanosine triphosphate
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DRE	Drug regulatory elements
ds	Double stranded
d(T)	Deoxythymidine
DTT	Dithiothreitol

dTTP	Deoxythymidine triphosphate
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycol tetraacetic acid
ER	Endoplasmic reticulum
ETOH	Ethanol (ethyl alcohol)
g	gram
HPLC	High pressure liquid chromatography
HRP	Horse radish peroxidase
IgG	Immunoglobulin G
ip	Intraperitoneal
IPTG	Isopropyl-beta-D-thiogalactopyranoside
K	Kilodalton
Kg	Kilogram
LB	Luria-Bertani
M	Mole
3-MC	3-Methylcholanthrene
mg	milligram
mM	millimole
mm	millimeter
M_r	Relative molecular weight
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
$NADP^+$	Oxidized nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
ng	Nanogram

NIH	National Institutes of Health
nm	Nanometer
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
P_I	Isoelectric point
Pnp	P-nitrophenol
Poly(A^+) RNA	Polyadenylated ribonucleic acid
Poly d(G)	Poly deoxyguanidine
ppt	Precipitate
R_f	Relative mobility
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate
SM	Sodium Chloride - Magnesium sulfate
Sp-Activity	Specific Activity
SS	Single stranded
SS-phenol	Salt saturated phenol
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TE	Tris-ethylene diamine tetraacetic acid
tRNA	Transfer ribonucleic acid
TTBS	Tween Tris Buffered saline

TYE	Tryptone yeast extract
U	Units
UDPGT	Uridine diphosphate glucuronyltransferase
uCi	Microcurrie
Umole	Micromole
ug	Microgram
v/v	Volume/volume
w/v	Weight/volume
w/w	Weight/weight
X	Times
x g	Relative centrifugal force
x-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactoside

CHAPTER I

INTRODUCTION

UDP-glucuronyltransferase (UDPGT, EC 2.4.1.17) is an integral component of the microsomal membrane (Hochman et al., 1981; Mackenzie, 1987). This enzyme exists as a heterogenous molecule (Dutton, 1980) consisting of a family of isozymes that are structurally related, but differ in their substrate specificities and responses to different inducers. Three forms of UDPGT having overlapping substrate specificities and different degrees of regulation by compounds such as phenobarbital and 3-methylcholanthrene (3-MC) have been identified in the rat (Bock et al., 1979), rabbit (Tukey and Tephly, 1981) and guinea pig (Hochman and Zakim, 1983). As a group of enzymes, UDPGTs are difficult to solublize, and in general are quite unstable after partial purification. Therefore, until now, much of the data on substrate specificity has been obtained using crude homogenates. We know that UDPGT catalyzes the glucuronidation of a wide variety of endogenous and xenobiotic compounds (Boutin et al., 1984; 1985). For the present, our knowledge of the number of multiple forms and their substrate specificity is inadequate because of the difficulties involved in purification, isolation and resolution of the different isoforms.

Probably, multiple forms of the transferase have evolved specifically to metabolize endogenous compounds in various tissues, and each form of transferase so far isolated might be specifically involved in glucuronidating a single endogenous substrate.

General Overview of Xenobiotic Metabolism

Generally, xenobiotics are compounds that are exogenous to the normal metabolism of an organism and may include endogenously generated metabolic intermediates that the organism cannot utilize. A molecular understanding of xenobiotics metabolism and their interaction with living organisms is essential for the rational development of new therapies, for the determination of toxic hazards, and for the development of new biocides for agriculture and medicine.

Interaction with xenobiotics involves several steps:

(1) Exposure: The way in which an organism becomes part of an environment containing the foreign compound.

(2) Uptake: Involves the biochemistry of cell membranes in the portals of entry and the structure-function relationships in xenobiotic absorption.

(3) Distribution: Involves the mechanism of transport throughout the body and the distribution

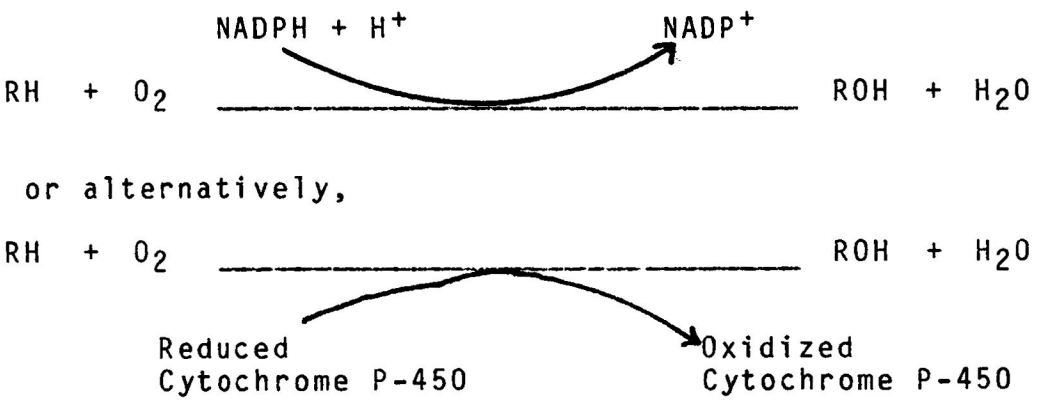
between tissues of the xenobiotic or its metabolites.

(4) Metabolism: Takes place at the portal of entry or in such organs as the liver and extrahepatic tissues, and involves the study of the enzymes that detoxify and/or activate xenobiotics (Hodgson and Guthrie, 1980).

The principal portals of entry for xenobiotics are the skin, the gastrointestinal tract, and the lungs (Nelson, 1961). In most cases, the foreign compound must pass through a number of biological membranes before it can be distributed throughout the body and uptake depends on the membrane as well as the physical properties of the xenogen. The lipophilicity of the cell membrane is the most important factor in the uptake of exogenous molecules (Castuma and Brenner, 1986) and active transport or pinocytosis is not as common as diffusion in the uptake of xenogens across lipid membranes. Furthermore, the majority of xenobiotics that enter the body are lipophilic (Eisenberg and McLaughlin, 1976).

The metabolism of xenobiotics, which is carried out by a wide range of relatively nonspecific enzymes, serves to increase their water solubility and make possible their elimination from the body (Conney, 1967). The process of

biotransformation consists of two phases. In Phase I, a reactive polar group is introduced into the molecule, rendering it a suitable substrate for Phase II reaction (Magdalou et al., 1982). The Phase I reactions which activate the xenobiotic include the well-documented cytochrome P-450-dependent mixed function oxidations as well as reductions and hydroxylations. Since the electrons required in the reduction of cytochrome P-450 are derived from NADPH, the overall reaction can be written as follows (where RH is the substrate):



On the other hand, Phase II reactions include all the conjugation reactions in which a polar group on the xenogen is combined with an endogenous compound such as glucuronic acid, glutathione, sulfate, glycine and glutamate to form a highly water-soluble conjugate that can be eliminated from the body. Although the liver is the most studied organ with regard to xenobiotic metabolism, several other organs are

known to be active in this respect; however, neither the specific activity nor the range of substrates metabolized is as large as in the liver. An organ such as the kidney, may also be an important site for xenobiotic metabolism (Bock et al., 1984). The distribution of xenobiotic metabolizing enzymes among organs is still a subject for more investigation.

Because foreign compounds are both activated and inactivated metabolically, physiological factors affecting metabolic rates can have dramatic effects on the induction of gene expression by the xenogens. These factors include sex, age, pregnancy and diet as well as route of administration (Conney et al., 1977; Wang et al., 1986).

Xenogens can be substrates, inhibitors, or inducers of the enzymes that metabolize them and, not infrequently, serve in more than one of these roles. Since the enzymes in question have multiple substrates, numerous interactions between foreign compounds are possible. These may be synergistic or antagonistic and may have a profound effect on the metabolism of the xenogen (Sanvordeker and Lambert, 1974). Depending upon the compounds and the enzymes involved in a particular interaction, the effect can be an increase or decrease in gene expression.

The cell type that has been studied most extensively in association with xenobiotic metabolism is the hepatocyte, the cell that forms the bulk of the liver. These cells are highly active metabolically, both in normal intermediary metabolism and in reactions involving xenobiotics (Chen and Andrade, 1976).

The action of xenobiotics has also been demonstrated in the nucleus. The chromosomes which contain the DNA responsible for the code for most of the proteins synthesized in the cell is the site for the primary reaction in carcinogenesis, since some carcinogens covalently bind to DNA (Nebert and Felton, 1976). Depending upon the xenogen, the organ, and the cell type involved, similar reactions are involved in mutagenesis and other reproductive effects as well as in teratogenesis (Hucker, 1970). The nuclear envelope has recently been shown to have an active aryl hydrocarbon hydrolase (a component of the cytochrome P-450 system). Both the rough and smooth endoplasmic reticulum are active in the oxidation of xenobiotics; usually the smooth ER has the highest specific activity (Bochard et al., 1975).

Following phase I enzyme activation of a xenobiotic in the body, the metabolite ultimately arrives at a site of action such as the nucleic acid and proteins, or is conjugated with a hydrophilic compound by the deactivating enzymes.

Although almost any organ can be affected by the xenogen, some are more easily affected than others by particular classes of xenogens, and some have been studied in greater detail than others. Some of these responses seem to be receptor mediated. In all cases, however, xenogen-receptor interactions are of importance, and actively investigated. Some xenogens tend to affect either oxidative metabolism, the synapses of the nervous system, or the neuromuscular junction (Smith, 1978). The most common modes of action of xenogens involve interaction with nucleic acids, causing carcinogenesis or reproductive effects (Leonard and Jacob, 1977). Many metabolic pathways are affected by xenogens. These include glycolysis, the tricarboxylic acid cycle, the pentose cycle, the electron transport system and oxidative phosphorylation, nucleic acid synthesis, protein synthesis, as well as specialized systems as photosynthesis in plants (Yu, 1977). For instance, some of the xenobiotics can act as uncouplers of oxidative phosphorylation such as dinitrophenols (Dills and Klaasen, 1986), and others can alter the expression of the enzymes involved in intermediate metabolism such as acetaminophen that increases blood alanine amino-transferase levels (Hjelle et al., 1986). Either the unmetabolized xenogen or its metabolic products are ultimately excreted, the latter usually as conjugated products

resulting from Phase II reactions. The two primary routes of excretion include the urinary system and the bilary system (Hodgson and Guthrie, 1980). Minor routes include the lungs, sweat glands, sebaceous glands, hair, feathers and nails (Hodgson and Guthrie, 1980). Sex-related routes such as milk, eggs and fetus also contribute to xenobiotic excretion (Kitchin and Ebron, 1983).

Glucuronidation as a Detoxification Mechanism

Glucuronidation, catalyzed by UDP-glucuronyltransferase, represents one of the major biotransformation processes involved in the elimination of endogenous and xenobiotic compounds from the body (Dutton, 1980). This process involves the conjugation of compounds to UDP-glucuronic acid, which renders them more water-soluble and thus more easily excreted from the body. Substrates for glucuronidation include compounds possessing a hydroxyl, an amino, or a carboxylic acid group. Most of the substrates of UDPGT are the metabolites formed after a compound has been biotransformed by the Phase I enzymes (e.g. cytochrome P-450), specific hydrolases, N-oxidases and epoxidases. Both Phase I and II biotransformations take place mainly in

the liver, though these enzymes have been found in extrahepatic tissues such as lung, spleen, kidney and small intestine (Wishart, 1978; Schwenk and Locher, 1985).

The conjugation of numerous chemicals of diverse structure is believed to be accomplished through the selective induction of distinct forms of UDPGT (Bock et al., 1982).

The three most common types of glucuronic acid conjugations involve the reaction of the co-substrate uridine diphosphate glucuronic acid (UDPGA) with an aglycone (Fig. 1).

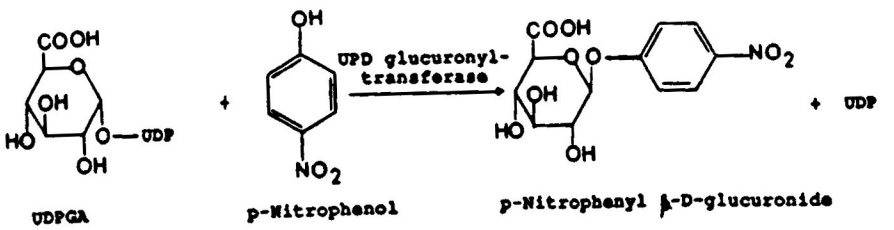
O-Glucuronides: This type of glucuronidation occurs with primary, secondary, and tertiary alcohols and phenols.

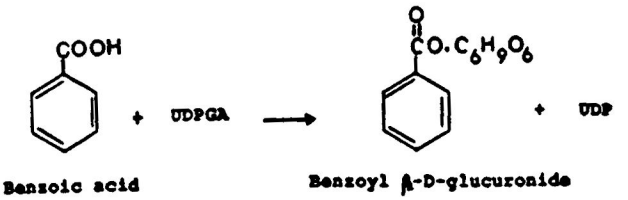
N-Glucuronides: Here the glucuronyl moiety attaches to the nitrogen of the aglycone with one of the following functional groups: aromatic amino group, sulfonamide group, carbamyl group, or heterocyclic nitrogen group.

S-Glucuronides: A number of thiol compounds are conjugated with glucuronic acid. S-glucuronidation is not very common, but is similar to N-glucuronidation in that the glucuronides formed are acid labile.

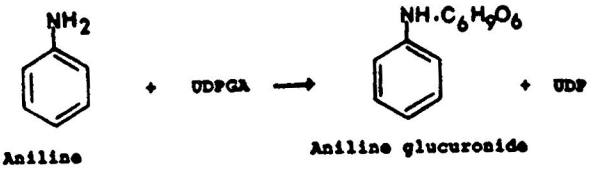
Fig. 1. Illustration of the glucuronidation reactions: Glycoside bond formation may occur with O, N, S and C atoms, forming the glucuronide.

1. O - Glucuronidation

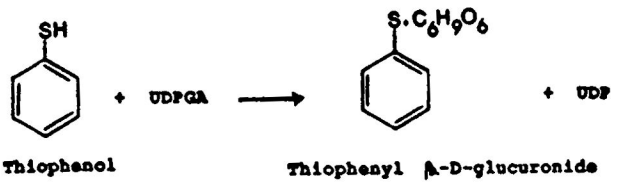




2. N - Glucuronidation



3. S - Glucuronidation



It has been shown through substrate specificity studies that some xenobiotics, such as 3-methylcholanthrene and phenobarbital, and some endogenous compounds, such as glucocorticoids, induce distinct forms of this enzyme (Bock et al., 1984). Additional support for the heterogeneity of UDPGT is the reported separation of distinct enzyme forms from rat and mouse liver via chromatography or chromatofocusing procedures (Tukey and Tephly, 1981).

The phenolic antioxidant butylated hydroxyanisole has also been shown to stimulate UDPGT activity in mammalian tissues (Moldeus et al., 1982) and has been reported to induce specific molecular forms of this enzyme (Sato et al., 1984). These observations are important because of the prevalence of this antioxidant in the human environment (mainly as a food and drug additive) and because some investigations have shown that it inhibits some of the harmful effects of other xenobiotics such as their toxicity, carcinogenicity and mutagenicity (Salocks et al., 1981; Miranda et al., 1981).

Because of the biological significance of UDPGT, it is important to determine the molecular basis of its multiplicity and the interrelationship(s) among the different forms. This will in turn assist in the elucidation of the mechanisms involved in the regulation of UDPGT gene(s).

CHAPTER II

REVIEW OF LITERATURE

Physical and Functional Characteristics of P-nitrophenol UDPGT.

SDS gel electrophoresis has revealed subunits from 50,000 to 57,000 daltons for p-nitrophenol UDP-glucuronsyltransferase (Tukey and Tephly, 1981; Peters and Jansen, 1988). This enzyme has a half-life of 18 to 20 days when stored at 0°C, and the addition of small phospholipid vesicles considerably improves the stability of the transferase. Storage by freezing at -20°C in 20% (w/v) glycerol results in a complete loss of activity. The enzyme is also thermolabile at 37°C for longer than 15 min. Extensive vacuum dialysis results in a loss of up to 40% of enzyme activity (Tukey et al., 1981). The purified transferase appears to exhibit absolute specificity toward UDP-glucuronic acid as the sugar nucleotide.

Native polyacrylamide gel electrophoresis has revealed a polypeptide of about 220,000 daltons that is believed to be a tetramer. This polypeptide resolves to 3 close bands on a two-dimensional gel (Bock et al., 1984), and has been postulated to have identical subunits, Mackenzie (1986).

The enzyme has a pH optimum for catalytic activity of 7.5 and an isoelectric point of 6.8. It has been shown to have 38% hydrophobic amino acids (Falany et al., 1986) and conjugates p-nitrophenol, estrone, and estradiol (Burchell and Weatherill, 1981).

Since the molecular weights of estrone and p-nitrophenol UDP-glucuronyltransferases isolated from the same tissue and animal are similar (Tukey et al., 1982), analysis of purity based on a single staining protein band may not be a final proof of homogeneity. However, these transferases can be separated based on their substrate selectivity, pH optima, isoelectric point, amino acid composition, and peptide fragment profiles after limited proteolysis (Tukey et al., 1982).

Rat liver UDPGT has been shown to resist a temperature of up to 50°C (Norling and Hanninen, 1974) and the glucuronide biosynthesis increases exponentially from 30°C to 44°C.

Purification and Assay:

Two procedures have been developed to separate and purify the enzyme that catalyzes glucuronidation of p-nitrophenol by ion-exchange chromatography (Burchell et al., 1981). Irrespective of the procedure adopted, the enzyme

is further purified by passing the ion exchange column fraction(s) through a UDPGA-hexanolamine - Sepharose 4B affinity column and eluting with the purification buffer containing 5 mM UDPGA. In addition, UDPGT has been purified by chromatofocusing followed by affinity chromatography. Homogeneity is assessed using SDS-polyacrylamide gel electrophoresis stained with Coomassie Brillant Blue.

The enzyme has been assayed either spectrophotometrically or radiometrically. Spectrophotometrically, UDPGT is assayed by quantitation of the disappearance rate of the substrate at 400 nm wavelength (Falany et al., 1986).

Radiometrically, the enzymes is assayed by monitoring the decrease in radioactivity of labelled substrate with respect to time (Whitmer et al., 1985).

UDPGT Kinetics and Enzyme Mechanisms:

UDP-glucuronic acid is the required cosubstrate for all glucuronidation reactions in mammals except cats where no UDPGT activity has been demonstrated (Hodgson et al., 1980). It has been shown that the rate of xenobiotic glucuronidation is dependent on the availability of UDP-glucuronic acid (Singh et al., 1981).

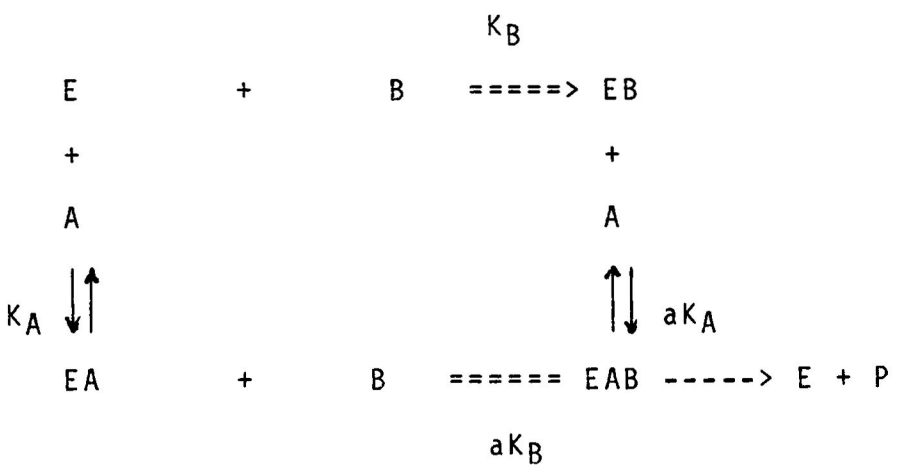
UDP-glucuronyltransferase exhibits functional heterogeneity according to the aglycone or substrate (Antoine

et. al. 1983) and the inducing agent (Wishart, 1978) used. UDP-glucuronyltransferase activities have been categorized into three major groups based on preferential induction of activities towards particular substrates by prototypic inducers. For instance, it has been shown that phenobarbital treatment enhances activities for one group of substrates typically represented by 2-hydroxybiphenyl, 4 hydroxybiphenyl, phenolphthalein and morphine. On the other hand, 3-methylcholanthrene treatment enhances activities towards substrates typically represented by p-nitrophenol (PNP), 3-hydroxybiphenyl and naphthol (Mackenzie et al., 1985).

Analysis of UDPGT affinity towards aglycones of each group and towards UDP-glucuronic acid in subcellular membranes could afford more evidence for the existence of different molecular forms of UDPGT which would account for the differences in substrate specificity. Antoine et al. (1984) have shown that in the endoplasmic reticulum, the conjugation of group 1 substrates (e.g., 4-nitrophenol) was at least twice that for group II aglycones (e.g. 1-borneol) or of an endogenous substrate (e.g., testosterne). In the golgi and plasma membrane, the different types of aglycones are metabolized to the same extent.

The kinetic mechanism of UDPGT activity is a rapid equilibrium, random bireactant system. Thus, there are two binding constants for each substrate, one for binding of the first substrate to the free enzyme (K'_{UDPGA} and $K'_{aglyone}$) and the other for binding of the second substrate to enzyme already saturated with the first substrate (K_{UDPGA} and $K_{aglycone}$). The kinetic properties of UDPGT are regulated via interactions between the enzyme and the phospholipids of the membrane (Hochman et al., 1981).

Proposed reaction and kinetic schemes for a rapid equilibrium, random order mechanism, such as in UDP-glucuronyltransferase catalysis, is as follows (Hochman et al., 1981):



E = UDPGT

A = p-nitrophenol or any other substrate (aglycone)

B = UDP glucuronic acid

K_A = Kinetic constant for UDPGT and p-nitrophenol association

K_B = Kinetic constant for UDPGT and UDPGA association

aK_A = Kinetic constant for p-nitrophenol association with enzyme-UDP-glucuronic acid complex

aK_B = Kinetic constant for UDPGA and p-nitrophenol-enzyme complex association

The dependence of the glucuronidation rate on UDP-glucuronic acid concentration in vivo or in isolated cells is consistent with the observation that the K_m of rat liver UDPGT for UDP-glucuronic acid ranges from 200 to 500 uM when the animal is treated with a xenobiotic. This concentration is above the hepatic UDP-glucuronic acid levels (250-350 uM) observed in untreated rats (Falany and Tephly, 1983).

The role of UDP-glucuronic acid as the limiting substrate in glucuronidation has implications in the area of capacity-limited conjugation (Hjelle, 1986). UDP-glucuronic acid is synthesized in the cytoplasm from UDP-glucose by UDP-glucose dehydrogenase, as an irreversible, NAD-requiring reaction. UDP-glucuronic acid and NADH inhibit the

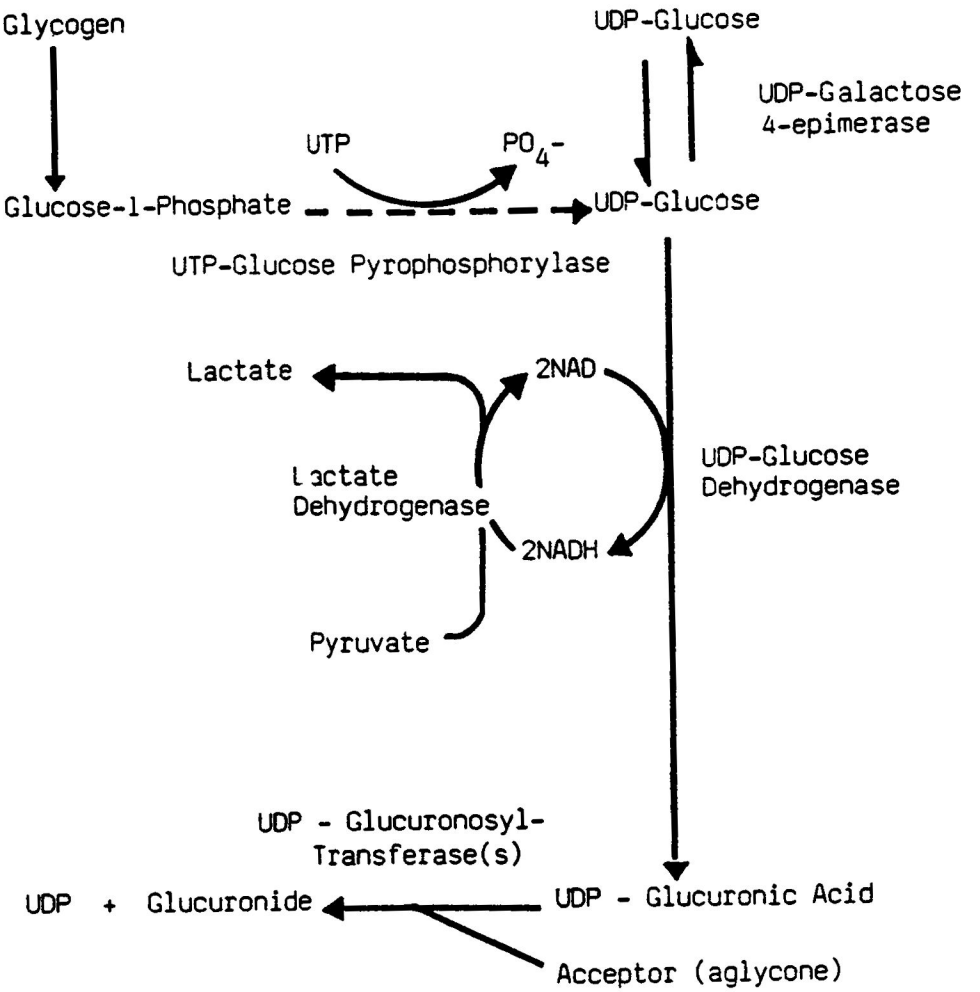
dehydrogenase which ultimately regulates the concentration of available UDP-glucuronic acid (Fig. 2).

Effects of Phospholipid on UDPGT Activity

It has been proposed that the kinetic properties of UDPGT are regulated via interactions between it and the phospholipids of the membrane (Zakim et al., 1975). This idea was experimentally demonstrated by altering the composition and/or structure of the lipid portion of the microsomal membrane. In the study, changes in the kinetic properties of UDP-glucuronyltransferase (Zakim and Vessey, 1975) were shown. Moreover, delipidation of a partially purified preparation of the enzyme leads to a marked diminution of activity, which can be restored by adding selected types of phospholipids (Gorski and Kasper, 1978).

The reconstitution of a delipidated form of UDPGT appears to require a phospholipid containing a phosphatidylcholine moiety (Erickson et al., 1978). The specific activity of the reconstituted enzyme is influenced by the length and unsaturation of the acyl groups of added phospholipids. UDP-glucuronyltransferase, thus, is one of several membrane-bound enzymes regulated by lipid-protein interactions.

Fig. 2. Enzymes, intermediates and cofactors involved in UDP-glucuronic acid synthesis and xenobiotic glucuronidation (Hjelle, 1986).


Glycogen
UDP-Glucose
UDP-Galactose
4-epimerase
UTP
PO_4^-
Glucose-1-Phosphate
UDP-Glucose
UTP-Glucose Pyrophosphorylase
Lactate
2NAD
Lactate
Dehydrogenase
UDP-Glucose
Dehydrogenase
2NADH
Pyruvate
UDP - Glucuronosyl-
Transferase(s)
UDP + Glucuronide
UDP - Glucuronic Acid
Acceptor (aglycone)

Inhibitors and Stimulators of UDPGT.

In vitro, the activity of UDP-glucuronyltransferase has been modulated by the addition of phospholipids, especially phosphatidylcholine derivatives (Whitmer et al., 1985). Pogell and Leloir (1961) described the stimulation of glucuronyltransferase activity in rat liver microsomes by the addition of adenosine triphosphate (ATP), UDP-N-acetylglucosamine (an allosteric effector) and albumin. Diethylnitrosoamine and alkyl ketones also activate the purified enzyme two to three fold when 2-aminophenol was used as substrate (Burchell and Weatherill, 1981). According to Burchell and Weatherill (1981), transferase activity also increases approximately 30% by the addition of 2 to 20 mM magnesium ion.

The sulfhydryl compounds p-hydroxymercuric benzoate and p-chloromercuriphenyl sulfonate inhibit glucuronyltransferase activity. The divalent cation Ca^{2+} at a concentration greater than 0.5 mM inhibits p-nitrophenyl glucuronide synthesis, which is reversed by adding 10 mM EDTA (Isselbacher et al., 1962). Dills and Klassen (1986) showed that compounds that impair cellular energy production such as cyanide and 2,4-dinitrophenol decreased UDPGT activity by the depletion of ATP in metabolically compromised cells. This results in decreased synthesis of the co-substrate UDP-glucuronic acid. Also, modulations in rat

hepatic microsomal UDPGT activity have been observed during carbon tetrachloride (CCl_4) poisoning, with a large decrease in the enzyme's cooperativity and an increase in the membrane fluidity, which may be an early key event in CCl_4 induced liver injury (Deliconstantines et al., 1986). In addition, anthranilic acid has been shown to competitively inhibit p-nitrophenol UDP-glucuronyltransferase (Isselbacher et al., 1962). The end products of the p-nitrophenol UDPGT reaction (UDP and p-nitrophenylglucuronide) also inhibit this enzyme (Vessey and Zakim, 1972). Recently Shipley and Weiner, (1987) showed that adenosine and dibutryl cAMP inhibit UDPGA accumulation and, ultimately, glucuronidation.

It has now been demonstrated that at least five rate controlling factors, (substrate supply, UDPGA levels, NAD^+/NADH redox state, carbohydrate reserve, and the activity of UDPGT) influence glucuronidation in intact cells. Conditions that alter the cellular level of these factors, obviously influence UDPGT activity.

Tissue and Intracellular Distribution of UDPGT

Considering that glucuronidation is an important pathway in detoxification (Dutton, 1980), tissue and intracellular location of UDPGT may be important in the chemical defense against toxins. Using purified IgG from rabbit antisera against one of the UDPGT isoforms that is known to

cross react with the other isoforms, the tissue and intracellular distribution of UDPGT in rat liver, kidney, jejunal mucosa and adrenal gland have been determined. This distribution agrees with the tissue distribution of the enzyme activity (Roychowdhury et al., 1985).

The kidney has been shown to contribute to glucurondiation of bilirubin in the bile duct of ligated rats and to 3 alpha-glucuronidation of estriol in humans (Mellor and Hobkirk, 1975). The digestive tract is a major portal of entry of toxins and carcinogens, and because glucuronidation by the gut markedly reduces the entry of several lipophilic substances, such as opiates from the gut lumen into the serum, the presence of the transferase in the mucosal epithelium may provide a barrier against the entry of toxins and carcinogens. (Rance and Shillingford, 1976). Glucuronidation of simple phenols occurs throughout the small intestine; isolated small intestinal mucosal epithelial cells have been reported to synthesize and glucuronidate bilirubin (Hartman and Bissell, 1982). The presence of UDPGT activity for simple phenols in the adrenal gland has been demonstrated. The low levels of the transferase activity for steroid substrates is consistent with the UDPGT activity observed in adrenal glands in vivo (Saez et al., 1972).

Studies on the distribution of UDPGT in subcellular fractions of tissue homogenates indicate that the transferase activity is concentrated in the microsomal fraction (Amar-Costec et al., 1974) and nuclear envelope (Beaufay et al., 1974). Less enzyme activity has been reported in fractions enriched in golgi apparatus (Hauser et al., 1984), mitochondria (Schumaker et al., 1972) and plasma membrane (Magdalou et al., 1984). Recently, immunoreactive UDPGT has been observed exclusively in the endoplasmic reticulum and nuclear envelope (Roychowdhury et al., 1985). Using the same immunocytochemical techniques, no detectable UDPGT has been observed in the golgi apparatus, lysosomes, canalicular or sinusoidal portions of the plasma membrane, and gap junctions.

Earlier, UDPGT had been demonstrated in these organelles and was claimed to be accessible to antibodies and immunoreagents (Spater et al., 1983). Previous reports of the presence of UDPGT activity in the golgi apparatus, mitochondria (Schumaker et al., 1972) and plasma membrane (Magdalou et al., 1982) may be due to contamination of these subcellular fractions by the endoplasmic reticulum. This disparity can further be explained by the differences in the assay method and substrates utilized by the investigators. UDPGTs are glycoproteins (Owens and Mackenzie,

1984), and the lack of immunodetectable transferases in the golgi system may indicate either the glycosylation of these proteins in other intracellular sites (Atkinson and Lee, 1984) or a low concentration of the transferases that is undetectable by the techniques employed.

Xenobiotic Induction of UDPGT

The induction of drug metabolizing enzymes in response to xenobiotic administration has been well documented. Several classes of compounds which include polycyclic aromatic hydrocarbons, barbiturates and steroid hormones have been identified as inducers of detoxifying enzymes (Castren and Oikari, 1983). Unlike most inducers, 3-MC induces both microsomal cytochrome P-450 and UDPGT (Group 2) concomitantly, probably through a common receptor protein (Lilienblum et al., 1982). Recently, Finley et al. (1986) and Sweeney and Reinke, (1987), confirmed previous studies that consumption of ethanol greatly enhances the induction of p-nitrophenol UDPGT as well as many other biotransformation enzymes. Fischer and Schauer (1985) have shown that prolonged use of oral contraceptives increases UDPGT in putative preneoplastic foci in human liver which may be of prognostic significance in women that use oral contraceptives.

Effects of Butylated Hydroxyanisole on Cellular Metabolism and UDPGT Expression

BHA is a food antioxidant that possesses a variety of properties of toxicological interest as it prevents oxygen induced lipid peroxidation (Williams et al., 1986). This compound has been shown to reduce carcinogenic and hepatotoxic effects of several structurally diverse compounds such as polycyclic hydrocarbons, halogenated solvents, drugs and plant toxins (Wattenberg, 1972; Miranda, et al., 1981). The protective effects of BHA are thought to be largely due to its ability to increase the activities of several enzymes involved in the detoxification of various toxic or carcinogenic metabolites. BHA increases hepatic and extrahepatic activities of glutathione S-transferase(s) and epoxide hydrolase and elevates nonprotein sulfhydryl content (Cha and Bueding, 1979; Stewart and Boston, 1987). It, therefore, has been postulated that the concentration of reactive and potentially harmful metabolites of polycyclic hydrocarbons, bromobenzene, monocrotaline, and other compounds is decreased as a consequence of these biochemical changes.

Recently, an increase in an additional phase II biotransformation pathway has been reported to occur in mice following BHA feeding. Using 4-nitrophenol, 4-aminophenol, and 1-naphthol as substrates, the activity of mouse hepatic

UDP-glucuronyltransferase was significantly increased following BHA treatment (Cha and Bueding, 1979). BHA has also been shown to enhance the activity of UDP-glucose dehydrogenase (Cha and Bueding, 1982).

The biochemical mechanism by which butylated hydroxyanisole increase the glucuronidation of xenobiotics has been examined (Hazelton et al., 1985). Hepatic UDP-glucuronyltransferase activity increases toward specific substrates in native and detergent activated microsomes. In general, the increase is toward group 1 substrates (1-napthol and p-nitrophenol), whereas no changes are found with group 2 (choramphenicol) or group 3 (digitoxigenin monodigitoxoside) substrates. However, Stewart and McCrary (1987) recently showed that BHA and 3-MC induced phenolphathalien (another group 3 substrate) UDPGT by 2-fold. BHA treatment increases UDP-glucuronic acid content by increasing UDP-glucose concentration and enhancing UDP-glucose dehydrogenase activity (Ordman and Kirkwood, 1977). BHA, like 3-MC, has been shown to have a more pronounced effect in female than male mice (Hazelton et al., 1985; Astrom et al., 1986). These observations suggest that BHA increases the capacity for glucuronidation by elevating both UDP-glucuronsyltransferase activities and UDP-glucuronic acid concentration in liver.

Investigation of induction of drug metabolizing enzymes, especially of the detoxicating enzymes, has shown that specific molecular forms of UDP-glucuronyltransferase become apparent in hepatic preneoplastic cell populations (Sato et al., 1984). Recently, there are reports implicating BHA (which induces some of these UDPGT forms) as a cancer causing agent in the fore-stomach of rats and hamster (Ito et al., 1983) and as a promoter of urinary bladder carcinogenesis (Imaida et al., 1983). Therefore, it implies that some of these BHA induced specific forms of UDPGT may serve as markers for pre-neoplasm in some animals. The appearance of forestomach tumors in rats is particularly troublesome in evaluating potential chemical hazard, since the target site, the rat forestomach, is absent in man. However, BHA-associated forestomach tumors appear as early as 3 months in hepatoectomized rats, whereas in intact rats the appearance of these tumors in seen after a prolonged latent period of 15-20 months (Abraham et al., 1986). These opposing effects of BHA could be due to reduced activation of the carcinogen in the liver and increased excretion in the urine of deconjugated metabolites that

affect the bladder. The possible enhancement of carcinogenicity by BHA represents a serious complication of any anticipated use of this compound to prevent human cancer (Williams, 1984).

Pathological Aspects of UDPGT

Some pathological conditions have been attributed to inadequate expression of UDPGT. Deficient hepatic UDPGT activity has been demonstrated in (a) Gunn rats (Dutton, 1980), (b) Crigler-Najjar syndrome (type I) patients (Wolkoff et al., 1983), (c) Crigler-Najjar syndrome (type II) patients (Arias et al., 1969), (d) Gilbertis syndrome in man (Wolkoff et al., 1983), (e) Gilbertis syndrome in Bolivian squirrel monkeys (Roychowdhury et al., 1982), and (f) the most common form of deficiency, neonatal unconjugated hyperbilirubinemia in man and monkeys (Gartner et al., 1977). In these disorders, deficiency in hepatic UDPGT activity has been demonstrated when homogenates of microsomes were incubated with UDPGA and bilirubin or several other glucuronide acceptors (Roychowdhury et al., 1982; Wolkoff et al., 1983).

Until advanced techniques in UDPGT purification became available, limited answers were available on the homogeneity, substrate specificites, effects of microsomal membrane

phospholipids as well as the specific structural and molecular defects in disorders characterized by deficiency in UDPGT activity. Deficiency in hepatic UDPGT function in humans is readily detected (Roychowdhury et al., 1984) since affected individuals have non-hemolytic unconjugated hyperbilirubinemia which is usually severe enough to produce clinical jaundice. Pathological conditions such as hyperthyroidism, starvation and experimental diabetes have been shown to decrease the formation of the obligatory cofactor UDPGA (Shipley and Weiner, 1987) and therefore, the formation of glucuronides. These conditions generally increase tissue cAMP via stimulation of adenylate cyclase. It is therefore possible that cAMP plays a role in the alterations seen in glucuronidation under these conditions.

Based on studies with Gunn rat liver microsomes, it has been suggested that the UDPGT deficiency results from an autosomal recessively inherited defect in the primary sequence of UDPGT gene(s) (Wolkoff et al., 1983). Clinical studies of the various forms of UDPGT deficiency reveal variation in glucuronidation of different acceptors in vivo and in vitro. In Gunn rats, for instance, there is a reduced rate of glucuronidation with phenols compared to aniline and steroid hormones (Jacobson et al., 1975), which are reported to be normal. In addition to the Gunn rat, it

has been shown that patients with Crigler-Najjar type II syndrome often have other family members who have Gilbert's syndrome (Wolkoff et al., 1983). It is possible that the different molecular defects in altered UDPGT involves one or more enzyme isoforms, (Irshaid and Tephly, 1987; Coughtrie et al., 1987) since it has been speculated that UDPGTs may be hetero-oligomers in which the various forms are reconstituted in vivo to form a family of enzymes with distinct but partially overlapping substrate specificity.

Some Molecular Aspects of UDPGT Activity

The molecular mechanisms involved in UDPGT regulation have rigorously been investigated in several laboratories. However, these investigations in mammalian tissues are complicated by the substrate specificity and range of measurable changes of transferase activities in response to inducing agents (Lillienblum et al., 1982). To circumvent some of these problems, Burchell et al. (1984) used the developing chick embryo in the study of the control of gene expression by drugs and hormones. The activity of hepatic UDP-glucuronyltransferase towards some substrates is virtually absent from chick embryo liver until hatching, when it rises suddenly to adult values.

In investigating the transcriptional and translational events involved in drug-mediated stimulation of UDPGT synthesis, in 1984 Burchell et al., observed that the precocious development of UDPGT in chick-embryo liver cultures was prevented by cycloheximide, which indicates that the increase of transferase activity was dependent on protein synthesis. Also, the group showed that mRNA specific for production of UDPGT accumulated (presumably by synthesis de novo) in chick embryo liver in response to phenobarbital.

Detailed analysis of the structure, transcription and genetic defects of UDPGT genes and the turnover of mRNA require cloned cDNAs coding for UDPGT. Jackson et al. (1984) for the first time used the expression vector Lambda gt11 as described by Young and Davis, 1983a,b to isolate cDNAs coding for UDPGT. Using these cDNAs, they have been able to estimate the size and content of UDPGT mRNAs. UDPGT mRNAs are of relatively low abundance in rat liver, less than 0.1% of the total mRNA (Burchell et al., 1985) if the animal is untreated.

Mackenzie et al., (1984a), by polysome immunoadsorption, have enriched for rat liver transferase specific mRNA and prepared cDNA sequences complementary to this mRNA. Using the recombinant DNA generated, they determined the size of the transferase mRNA and the effect of phenobarbital and

3-methylcholanthrene on the mRNA levels, as well as the sequence and expression of a cDNA encoding a phenobarbital-inducible form of UDPGT (Mackenzie, 1986). Recently, Iyanagi et al. (1986) cloned the 3-MC inducible form of p-nitrophenol UDPGT, and suggested conserved and variable regions in the UDPGT isoenzymes.

Research Justification and Goals

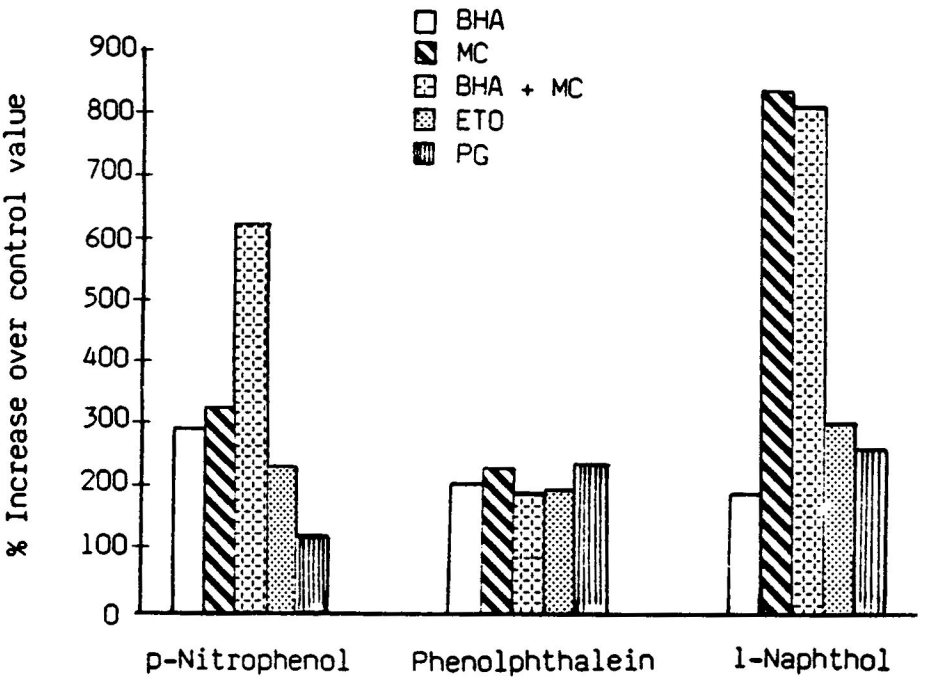
At the present time, we are yet to know if BHA and 3-MC induce different molecular forms of UDPGT, as well as the mode of expression of the UDPGT genes. However, we do know that a compound with potential carcinogenic effects (such as 3-methylcholanthrene, 3-MC) and a compound with "anti-cancer" properties such as (butylated hydroxyanisole, BHA) can induce UDPGT expression in mammals. In addition, the effect of a simultaneous induction is yet unknown even though this occurs always in human populations by choice or accident. Studies in our laboratory have attempted to determine if there are any differences in terms of cellular concentration, substrate specificity and kinetic parameters that exist in the enzyme isolated from rats treated with these xenobiotics (Stewart and McCrary, 1987).

From the data obtained, using p-nitrophenol as a substrate, it seems that BHA and 3-MC have the same stimulating effect. However, when animals were treated with both inducers simultaneously, an additive response was observed. This may suggest that these two inducers use different mechanisms to increase the activity of UDPGT. When phenolpthalien is used as the aglycone, and UDPGT activity measured, all the treatments gave the same response. Since the combined inducers had no more effect than either compound used alone, it is inferred that BHA and 3-MC may use the same mechanism to enhance phenolpthalien UDPGT activity (Fig. 3).

Based on UDPGT response to inducers (Wishart, 1978), the developmental patterns (Wishart, 1978) and tissue distribution (Bock et al., 1980) of glucuronidating activities, it seems that the forms of this transferase are regulated in a complex manner. Characterization of the regulation of this complex enzyme system can be accomplished by examining the mRNA and genes coding for these forms.

To date information concerned with the molecular mechanisms of UDPGT expression and genes coding for any transferase form or post-translational modifications are not available. Therefore, it is not presently clear if the different forms of UDPGT transferase are from different

Fig. 3. Effect of xenobiotic inducers on UDPGT activity toward various substrates (McCrary, 1987).


BHA
MC
BHA + MC
ETO
PG
% Increase over control value
900
800
700
600
500
400
300
200
100
0
p-Nitrophenol
Phenolphthalein
1-Naphthol

genes, or possibly result from the post-translational processing of a single gene product. This dilemma justified the investment of resources and effort in the project described here. In the long run, this study would be useful in the elucidation of the genetic, structural and functional interrelationships among the multiple forms of this enzyme.

The main objective of this investigation was to determine the interrelationship(s) between the UDPGT induced by the carcinogen 3-MC and the antioxidant BHA, so that ultimately we can understand the mechanism(s) of induction and regulation of UDPGT and other drug metabolizing enzymes. The hypothesis was that BHA and 3-MC activate different genes that express different molecular forms of the enzyme UDPGT. Because of the multiplicity in the substrate specificity of the enzymes obtained from xenobiotic induction, this investigation was narrowed down to the enzyme forms that conjugate p-nitrophenol. Here, <u>in vitro</u> translation, cDNA cloning and specific immunoadsorption were utilized to measure the relationship among the transferases following butylated hydroxyanisole and 3-methylcholanthrene treatments.

Specifically, this study was designed to accomplished the following goals:

1. Purify to apparent homogeneity UDPGT from 3MC, BHA and 3 MC + BHA treated rat livers.

2. Raise antisera against p-nitrophenol UDPGT from the livers of treated rats, and determine immunological identity.

3. Isolate and characterize p-nitrophenol UDPGT specific mRNA by:

   a) *In vitro* translation of mRNA from the different treatment groups.

   b) Immunoprecipitation of the UDPGT using specific antisera.

   c) Generation of p-nitrophenol UDPGT cDNA.

   d) Cloning of cDNA into plasmids.

   e) Screening total rat liver cDNA library to isolate p-nitrophenol UDPGT carrying clones.

CHAPTER III

MATERIALS AND METHODS

Materials

The female Sprague-Dawley rats used in this investigation were purchased from Charles River Breeding Laboratories (Wilmington, MA) or bred and provided by Atlanta University Center, Laboratory Animal Resources. New Zealand white rabbits were purchased from Hazelton Lab., Denver, PA. The animal diets with and without butylated hydroxyanisole were purchased from Ralston Purina Co. (St. Louis, Mo). All animals were housed at the Atlanta University Center Science Research Institute Building (SRI). The compounds uridine disphosphate glucuronic acid (UDPGA), UDPGA-agarose affinity resin and para-nitrophenol (p-Np) were purchased from Sigma Chemical Co. (St. Louis, MO), and 3-methylcholanthrene was purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin). All animals were maintained on hardwood bedding, had access to food and water ad libitum, and were kept on a 12 h light-dark cycle. Goat anti-mouse UDPGT was generously provided by I. Owens of the National Institutes of Health, Bethseda, MD. The Lambda gt11 rat liver library was obtained from Clontech Labs, Palo Alto, CA.

Animal Treatment

There were four treatment groups of at least ten rats each: untreated; butylated hydroxyanisole treated; 3-methylcholanthrene treated; and butylated hydroxyanisole plus 3-methylcholanthrene treated groups. On arrival from the breeders, the rats were kept on an antioxidant free diet (Control diet) for at least one week. The treatment procedure was a modification of that described by Fouarge (1986). In this study, all BHA-treated rats fed on chow that contained 0.75% (w/w) BHA for two weeks. The 3-MC-treated rats received two injections of 3-MC in corn oil (ip), 40 mg/kg body weight on consecutive days. The BHA + 3-MC treatments was a combination of the above two treatments. At the end of each treatment period, the rats were starved for 24 h prior to sacrifice by decapitation with a guillotone.

Extraction of Microsomes from Rat Liver Homogenates.

The method described by Tukey et al., (1982) was employed here. The livers were quickly excised from each group of sacrified rat, pooled, minced and homogenates (33% w/v) prepared using 0.05 M Tris-HCl, pH 7.2, containing 0.15 M KCl, and 10% glycerol. Hepatic microsomes were

isolated by centrifugation. The initial centrifugation was at 9000 x g for 10 min. The supernatant was collected and centrifuged at 105,000 x g for 90 min. The supernatant was aliquoted at 0.5 ml per vial and stored at -80°C for other studies. The microsomal pellet was washed with 1.15% KCl (w/v), then resuspended in 0.05 M Tris-HCl, pH 7.2, containing 20% glycerol and 1.0 mM EDTA, aliquoted at 20 mg protein per 0.5 ml and stored at -80°C until needed. The amount of protein was determined by the method of Bradford (1976).

Enzyme Isolation and Purification

The microsomes were solubilized with the nonionic detergent Lubrol PX (10%, w/w), stirred at 4°C for 60 min and centrifuged at 105,000 x g for 90 min. The supernatant was collected and applied to a DEAE-cellulose column (Whatman 52) that was equilibrated with 0.05 M Tris-HCl, pH 8.0. The column was eluted with the purification buffer, 25 mM Tris-HCl, pH 7.4 containing 0.05% Lubrol PX, 0.1 mM 2-mercaptoethanol as described by Singh et al., (1981), followed by a 0-500 mM KCl linear gradient elution. Using a modification of the methods of Singh et al., (1981); Tukey and Tephly (1981); Tukey et al., (1982) and Bock et al., (1979), the peaks with UDPGT activity were pooled, dialyzed against two changes of the purification buffer overnight at 1.50 liters

per change, then concentrated with an amicon ultra-filtration apparatus (cut off mol. weight of 10,000 daltons). The presence of protein in the elution fractions was determined spectrophotometrically at A_{280}. To the pooled and concentrated fractions $MgCl_2$ was added (up to 5 mM), followed by application to a UDPGA-agarose column that had been equilibrated with the purification buffer. The affinity column was eluted sequentially, first with salt free purification buffer, followed by purification buffer containing 50 mM KCl, and finally with the purification buffer containing 50 mM KCl and 5 mM UDPGA. Spectrophotometric A_{280} readings were obtained for all the fractions, and the peaks assayed for pNp UDPGT activity. SDS/polyacrylamide gel electrophoresis was performed on the peaks with transferase activity to ascertain the number of forms present and the homogeneity.

Enzyme Assay

With p-nitrophenol as the susbtrate, glucuronyl transferase activity was measured spectrophotometrically at 400 nm by modification of the method by Isselbacher et al. (1962). The incubation system consisted in addition to the enzyme (100 ug), of 0.25 mM p-nitrophenol, 10 mM EDTA, 5 mM UDP-glucuronic acid, 5 mM $MgCl_2$; 100 mM Tris-HCl, pH 7.4,

and 100 ug/ml phosphatidylcholine in a total volume of 0.5 ml. UDP-glucuronic acid was omitted in the control reactions.

The reaction mixtures were incubated at 37°C for 20 min and the reaction stopped by the addition of 1.0 ml of 3% trichloroacetic acid (w/v, TCA), and allowed to stand for 5 min on ice (Green et al., 1985). The reaction mixture was centrifuged at 1000 x g for 10 min and the supernatant solution added to 0.1 ml of 5 N NaOH. The absorbance was immediately measured in curvettes (1.0 mm light-path) at 400 nm in a Beckman DU-8 spectrophotometer. P-nitrophenol UDPGT activity was quantitated by the disappearance rate of p-nitrophenol (Control A_{400} - Expt. A_{400}). The molar absorption coefficient of pNp (18.8 x 10^3 cm^2/M at pH 9.0) was used to calculate the amount of p-nitrophenol conjugated.

SDS Polyacrylamide Gel Electrophoresis and Molecular Weight Determination

Purified UDPGT fractions from the different treatments were analyzed using 10% sodium-dodecyl sulphate (SDS) polyacrylamide resolving gels and 3% stacking gels. The gel and samples (15 ug of UDPGT) were prepared according to Laemmli

(1970). Using the Protean II Bio-Rad gel apparatus, the samples were electrophoresed for 9 h at 3 milliamps per slot. Staining and destaining of the gels followed the procedure described by Laemmli (1970).

Approximate molecular weights were determined by comparison of UDPGT migration to relative mobilities of Bio-Rad low molecular weight standard on Coomassie Brillant Blue stained gels (Weber and Osborne, 1968; Ochs et al., 1981). The molecular weight markers included phosphorylase b-94K, BSA-67K, ovalbumin-43K, carbonic anhydrase-30K, soybean trypsin inhibitor - 20K and alpha-lactalbumin - 14.4K.

Silver Staining of SDS/PAGE Slab Gels

The methodology was as recommended by the manufacturer, Bio-Rad in the Silver Stain Kit. Briefly, the gel was fixed for at least 20 min in 10% methanol-10% acetic acid (v/v), followed by two separate 30 min fixations in 10% ethanol-5% acetic acid (v/v).

The fixative was replaced by a 1:10 dilution of the oxidizer concentrate and the gel was allowed to shake for 10 min. The gel was washed three times for 10 min with deionized water. The washing was followed by the addition

of 1:10 dilution of the silver reagent concentrate for 30 min, and then washed for 2 min in deionized water. The water was discarded, and enough developer (32 g/liter) added to cover the gel. The gel stayed in the developer until the solution turned yellow or brown precipitate appeared. The developer was then poured off and fresh developer was added for 5 min. This process was repeated twice. The reaction was stopped with 5% acetic acid (v/v) for 5 min, before the gel was immersed in deionized water.

The bands were observed under an illuminator, and the gels stored in zip-lock bags.

Extraction of UDPGT from SDS/PAGE for Anti-sera Production

Bands of interest were visualized in stained preparative gels according to Falany et al., (1986). The gels were stained for no more than 30 min and destained for less than 1 h. The SDS/PAGE bands were recovered individually by mechanical-elution as described by Sakakibara et al. (1987). The 57K p-nitrophenol UDPGT was extracted from a 10% SDS/PAGE gel by cutting out the appropriate band and grinding in a mortar with pestle for homogenization in 25 mM Tris-HCl, pH 7.4 and 0.1 mM 2-mercaptoethanol as modified by Yudelevich et al, 1973. After centrifugation at low speed

the gel was re-extracted, and the combined supernatants were then passed through a millipore filter and eventually concentrated using an amicon ultraconcentrator. Protein concentration was determined by the Bradford method (1976).

Antibody Production and Immunological Characterization

Antibody was raised in rabbits against p-nitrophenol UDPGT from 3-MC treated rat livers, according to the methods of Pickett et al. (1981) and Singh et al. (1985). Prior to immunization with specific antigen (UDPGT), 10 ml of blood was collected from the ear vein of each rabbit using butterfly infusion needles. The blood was centrifuged at 2,500 x g and the pre-immune serum collected and stored at -80°C until needed. Following the pre-immune serum collection, about 200-300 ug of the purified enzyme in Freund's complete adjuvant in a total of 1.0 ml was injected intramuscularly at about 4 sites on the rabbit. Booster injections consisting of 100-200 ug of purified enzyme in incomplete Freund's adjuvant was given subcutaneously at approximately 7 day intervals for 8 weeks. From the third boost, 2 ml of venous blood was collected to check for antibody production by double immunodiffusion (Ouchterlony, 1968). By the seventh or eighth weeks post-immunization, the rabbits were bled out by cardiac puncture for immune sera collection as described by Mackenzie et al. (1984b).

Acepromazine (11 mg/kg body weight) and ketamine (22 mg/kg body weight) were used to anaesthesize the rabbits before sacrifice.

IgG was purified from the antiserum by ammonium sulphate fractionation followed by DEAE-cellulose chromatography and tested by double immunodiffusion (Ouchterlony, 1968) against the specific UDPGT used in its production as well as the UDPGT from other treatments to ascertain identity and cross-reactivity of the antigens. Furthermore, immunoblot analysis against purified transferases from the different treatments was performed. To evaluate the recognition of the functional forms and interrelationships among the UDPGT from the different treatments, purified antigens (100 ug) was gently stirred with the specific IgG. The immune complexes were removed after gentle mixing (overnight at 4°C) with a Staphylococcal protein A-sepharose slurry, followed by centrifugation (10,000 xg for 1 min). After washing the protein A-sepharose beads twice with 0.5 ml of 0.1 M Tris-HCl containing 50 mM KCl, the supernatants were pooled and UDPGT activity for p-nitrophenol was assayed (Roychowdhury et al., 1985). The study was repeated using the pre-immune serum for adsorption and inhibition.

Use of Dot-blot Assay to Titer Anti-UDPGT Serum

Partially purified, mechanically eluted, solubilized and unsolubilized microsomes containing p-nitrophenol UDPGT were dot blotted to a nitrocellulose membrane. The membrane was placed into a blocking solution (3% BSA in 20 mM Tris, 150 mM NaCl, pH 7.5). The solution was gently agitated for 30 min using a shaker platform. The blocking solution was removed and the membrane washed two times, 5 min each in TTBS (20 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.5) on a rocker platform. The wash solution was removed and different dilutions (0-1:1000) of the anti-UDPGT in TBS + 1% BSA was added. The reaction was allowed to continue for 2 h with gentle agitation. The antibody solution was removed and the membrane washed two times, 5 min each in TTBS on a rocker platform. The wash solution was removed and GAR-HRP (Goat anti-rabbit-Horse Radish Peroxidase conjugate), the second antibody, was added in the antibody buffer (1% BSA in TTBS). This reaction was allowed to proceed with gentle agitation for 2 h. The second antibody solution was discarded and the membrane washed two times, 5 min each in TTBS with shaking. The membrane was rinsed once for 5 min in TBS to eliminate residual detergent. The membrane was developed in color development solution (4-chloro-1-naphthol) for 30 min with gentle agitation. When

satisfactory signal (dark blue color) was generated, the membrane was removed and washed with water. Following the washing and drying steps, the membrane was scanned with a densitometer.

Western Blot Analysis

This assay was performed using a modification of the procedures suggested in Pucci et al. (1987) and the Bio-Rad manual. Solubilized microsome from 3-MC treated livers was electrophoretically separated on a 7.5% SDS/PAGE gel as described by Laemilli (1976). The protein bands in the polyacrylamide gel were electro-transferred to nitrocellulose filters overnight at 200 milliampere of current in a transfer buffer (20 mM Tris-HCl, pH 8.3 and 192 mM glycerine, 20% methanol) as recommended by Barlow et al. (1987). The filters were incubated in different dilutions of the rabbit anti-rat p-nitrophenol UDPGT serum. The incubations, washings and development of filters after reaction with the substrate is the same as that of the dot blot assay described above, except that the densitometric scan was omitted.

RNA Isolation and Purification

Total RNA from treated rat livers was isolated, extracted and purified using a modified guanidium thiocyanate

method of Chirgwin et al. (1979) and Spindler et al. (1984). Briefly, 10 g of minced liver tissue was homogenized in a solution containing 4 M guanidium thiocyanate, 0.5% sodium N-laurylsarcosine, 0.025 M sodium acetate, pH 7.0, 0.1 M 2-mercaptoethanol and 0.1% antifoam (Sigma). The RNA was freed of protein by ethanol precipitation or by sedimentation through 5.7 M cesium chloride (CsCl) followed by two cycles of phenol-chloroform extraction and etherization. The RNA samples were stored in 70% ethanol at -80°C until needed. Poly(A^+) RNA was isolated by oligo(dT) cellulose chromatography as described by Aviv and Leder (1972) and modified by Krajewska et al. (1986) with an intermediate heat step. Briefly, the column was prepared by resuspending 0.3 grams of oligo (dT) cellulose (Type 3) in the elution buffer (10 mM Tris-HCl, pH 7.5, 1.0 mM EDTA) and the column poured and packed by gravity. The column was washed with 10 bed volumes of the binding buffer (0.5 mM LiCl, 0.2% SDS, 20 mM Tris-HCl pH 7.4). The crude samples (total RNA) were heated for 2 min at 68°C in sterile water and then rapidly quenched on ice prior to the addition of an equal volume of 2X binding buffer and application to the column. Bound poly(A^+) RNA was eluted with 10 mM

Tris-HCl, pH 7.5 and 1.0 mM EDTA. The concentration of RNA was determined spectrophotometrically, assuming that an A_{260} of 1.0 is equivalent to 40 ug/ml of RNA.

cDNA Construction

Double-stranded cDNA was made from enriched transferase poly(A^+) RNA by the methods of Gubler and Hoffman (1983) and was inserted into the PstI site of pBR322-ampicillin sensitive, tetracycline resistant plasmids. Colonies were selected on TYE agar plates (10 g Tryptone, 5 g NaCl, 3 g KCl, 2 g yeast extract, 1.5 g Agar) containing 15 ug/ml of ampicillin and 15 ug/ml tetracycline. The synthesis of the first strand cDNA was carried out in a reaction volume of 60 ul containing 50 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$, 10 mM DTT, 4 mM sodium pyrophosphate, 1.25 mM dGTP, 1.25 mM dATP, 1.25 mM TTP, 0.5 mM dCTP, 50 uCi [^{32}P]ATP (approx. 800 Ci/mmol), 100 ug/ml oligo dT, 150 ug/ml poly(A^+) RNA and 3000 units of reverse transcriptase/ml for 30 min at 43°C. The reaction was stopped by adding EDTA to 20 mM. The products were extracted with phenol and precipitated with ethanol and 2 M ammonium acetate as described by Okayama and Berg (1982). The amount of first strand synthesized was estimated by assaying TCA-insoluble radioactivity.

For second strand synthesis, up to 500 ng of the single-stranded cDNA (i.e., 1 ug of hybrid) was processed in 100 ul of 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM ammonium sulfate, 100 mM KCl, 0.15 mM NAD, 50 ug per ml BSA, 40 uM dNTPs, 8.5 units per ml E. coli RNase H, 230 units per ml DNA polymerase 1 and 10 units per ml E. coli DNA ligase. The reaction mixture was incubated sequentially for 60 min at 12°C and 60 min at 22°C. Twenty millimoles of EDTA were added to stop the reactions. The products were extracted with phenol twice and then precipitated out of 2 M ammonium acetate with ethanol. The double stranded cDNA produced was then ready for tailing (using terminal transferase) and cloning without further sizing. Plasmid recombinants were constructed using the double stranded cDNA and pBR322.

Determination of cDNA Yield

The concentration of the actual nucleotide in 50 uCi of [^{32}P] and [^{35}S] or 200 uCi of [^{3}H] was calculated based on the specific activity (Ci/mMole) and other information supplied by the manufacturer. The concentration obtained was always less than 1.25 mM required for the experiment, the difference was made up with unlabelled nucleotides similar to the radioactive nucleotide in other to have equal concentration of the four nucleotides. The micromolar

concentration of the radioactive nucleotide was employed in calculating the specific activity for the first strand synthesis. Total radioactivity was determined by spotting 2 ul of the reaction mixture at time zero on a Whatman DE-81 filter paper, air drying and counting in 10 ml aqueous scintillation cocktail (Beckman Ready-Solv) in a Beckman scintillation counter.

The change in total counts (delta total count) was determined by the difference between precipitable counts in the reaction mixture at the end of reaction and at the beginning (time zero). Precipitation was done by spotting 2 ul of the reaction mixture on a Whatman DE-81 filter paper, air drying, soaking in ice-cold 10% Trichloroacetic acid (TCA) and chilling for about 30 min. The filters were air dried, washed by vacuum suction three times with 10% TCA, two times with 5% TCA, three times with 95% ethyl alcohol, and two times with 70% ethyl alcohol (five 5 min per wash). The filters were again air dried, solubilized in protosol at 55°C for 30 min before the aqueous scintillation cocktail was added. Radioactivity was counted on triplicate samples for 1 min each, and the mean counts per minute (CPM) was calculated.

Specific activity was then calculated by the formula below:

Specific Activity (CPM/ug RNA) =

$$\frac{\text{Total Count (cpm)}}{\text{Final concentration nucleotide with radioactivity (Umole)} \times 310 \times 4}$$

In the above formula three hundred and ten represents the average molecular weight of a nucleotide and four stands for the number of nucleotides.

The change in total counts and specific activity obtained above were used to determine the first strand cDNA yield as indicated below.

$$\text{cDNA yield (ug)} = \frac{\text{Change in total count}}{\text{Specific Activity}}$$

The efficiency of first strand cDNA yield was obtained by the following formula:

$$\text{Efficiency (\%)} = \frac{\text{First strand cDNA made (ug)} \times 100}{\text{Total mRNA used (ug)}}$$

To determine the second strand cDNA yield, the change in total count was determined as indicated for the first strand and yield calculated employing the specific activity obtained for the first strand as follows:

$$\text{Second strand cDNA yield (ug)} = \frac{\text{Change in total count (cpm)}}{\text{Specific Activity (CPM/ug)}}$$

Since the radioactive label was only on the first strand, the value obtained here was doubled to account for the double stranded nature of the cDNA at this point. Here, the percent recovery of cDNA was calculated by dividing the double stranded cDNA recovered with two times the first strand yield and multiplied by 100.

$$\text{ds cDNA Recovery (\%)} = \frac{\text{Double stranded yield} \times 100}{(\text{First strand yield}) \times 2}$$

The difference between 100 and the above value accounted for materials lost in processing the first strand cDNA to generate the second strand.

Construction of cDNA from Total mRNA for Cloning into pBR322

To amplify specific mRNA expression, the UDPGT enriched poly(A^+) RNA (2.5 ug) used to synthesize the double-stranded cDNA by reverse transcriptase came from BHA plus 3-MC-treated rat livers, which has been demonstrated to show additive UDPGT expression.

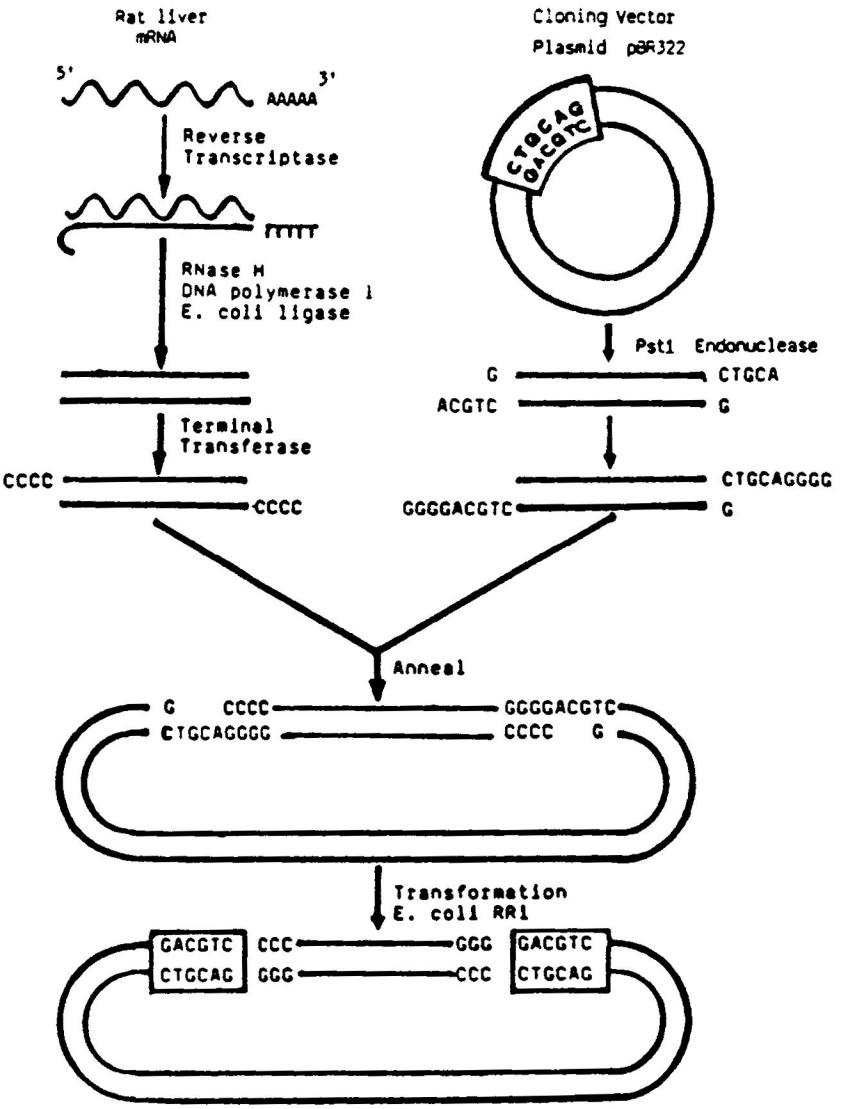
The blunt-ended cDNAs generated earlier were elongated with about 12-18 deoxycytidine residues per 3'hydroxyl end using terminal deoxynucleotide transferase. The tailed double stranded cDNA (100 ng) was annealed to 100 ng of pBR 322 cloning vector (New England Nuclear) at the PstI site as described by Mackenzie et al., (1984). Figure 4 shows how the

recombinant plasmid was used to transform E. coli HB101 by standard procedures (Maniatis et al. 1982; Okayama and Berg, 1982 and Gubler et al., 1983).

cDNA Construction from UDPGT Specific mRNA

To synthesize the first strand cDNA, poly (A^+) RNA (1.0 ug in 20 ul H_2O) was placed in a 1.5 ml microfuge tube after which 1.1 ul of 0.1 M methyl mercury hydroxide was added, and the mixture incubated for 10 min at room temperature. Later on, 2 ul of 700 mM beta-mercaptoethanol was added and incubated for 5 min at room temperature to inactivate the methyl mercury. In order, 5 ul of 1 M Tris-HCl, pH 8.7, 7 ul of 1 M KCl, 2 ul of 0.25 M $MgCl_2$, 10 ul of 1 mg/ml oligo (dT) (12-18), 5.0 ul of a solution containing 10 mM each: dGTP, dATP, dTTP, dCTP and 5 ul of alpha-[^{35}S]-ATP were added, followed by 2 ul RNasin (30 U/ul) and 4 ul AMV reverse transcriptase (10 U/ul). The reaction mixture was incubated for 2 h at 44°C, and 5 ul of 0.25 M EDTA, pH 8.0, added to stop the reaction. The reaction mixture was extracted with 70 ul SS-phenol: chloroform (1:1), mixed by vortexing, and the phases separated by centrifugation in a microcentrifuge for 2 min. The upper aqueous phase was

Fig. 4. Schematic diagram of cDNA construction for cloning into pBR322 plasmid. *E. coli* HB101 was transformed with the hybrid plasmid.


Rat liver
mRNA
5'
AAAAA
3'
Reverse
Transcriptase
RNase H
DNA polymerase I
E. coli ligase
Terminal
Transferase
CCCC
CCCC
Cloning Vector
Plasmid pBR322
CTGCAG
GACGTC
Pst1 Endonuclease
G
CTGCA
ACGTC
G
CTGCAGGGG
GGGGACGTC
G
Anneal
G
CCCC
GGGGACGTC
CTGCAGGGG
CCCC
G
Transformation
E. coli RR1
GACGTC
CTGCAG
CCC
GGG
GGG
CCC
GACGTC
CTGCAG

removed and extracted twice with 70 ul of chloroform; 60 ul of 4 M ammonium acetate were added to the aqueous phase and mixed thoroughly before 360 ul of 100% ethanol were added. This suspension was mixed, frozen on dry ice for 30 min or stored overnight at - 20°C. The cDNA sample was microcentrifuged for 10 min at 4°C, the supernatant was discarded, and the pellet washed with 150 ul of 80% ethanol that was kept cold but not frozen on dry ice. The microcentrifugation was repeated for 2 min at 4°C, the supernatant removed, and the pellet dried under vacuum. The pellet was resuspended in 50 ul of TE buffer (10 mM Tris-HCl, pH 7.4 and 0.1 mM EDTA, pH 8.0). Two ul of sample was precipitated with 10% TCA, as described earlier and the filter was counted to determine the amount of [^{35}S]-ATP incorporated into the first strand. The efficiency of mRNA copying was estimated.

To synthesize the second strand, 50 ul of the first strand mix was combined with 50 ul of 2 X second-strand buffer (8 ul 2 M Tris-HCl, pH 7.4, 4 ul 1M $MgCl_2$, 8 ul 1 M ammonium sulfate, 80 ul 1 M KCl, 4 ul nuclease-free BSA (10 mg/ml), 3.3 ul of a mixture of 10 mM each dGTP, dATP, dTTP, dCTP neutralized to pH 7.0 and 283 ul of H_2O, 1.0 ul of RNase H (2 U/ul) and 2.3 ul DNA polymerase 1 (5 U/ul) were added. The reaction mixture was incubated at 12°C for

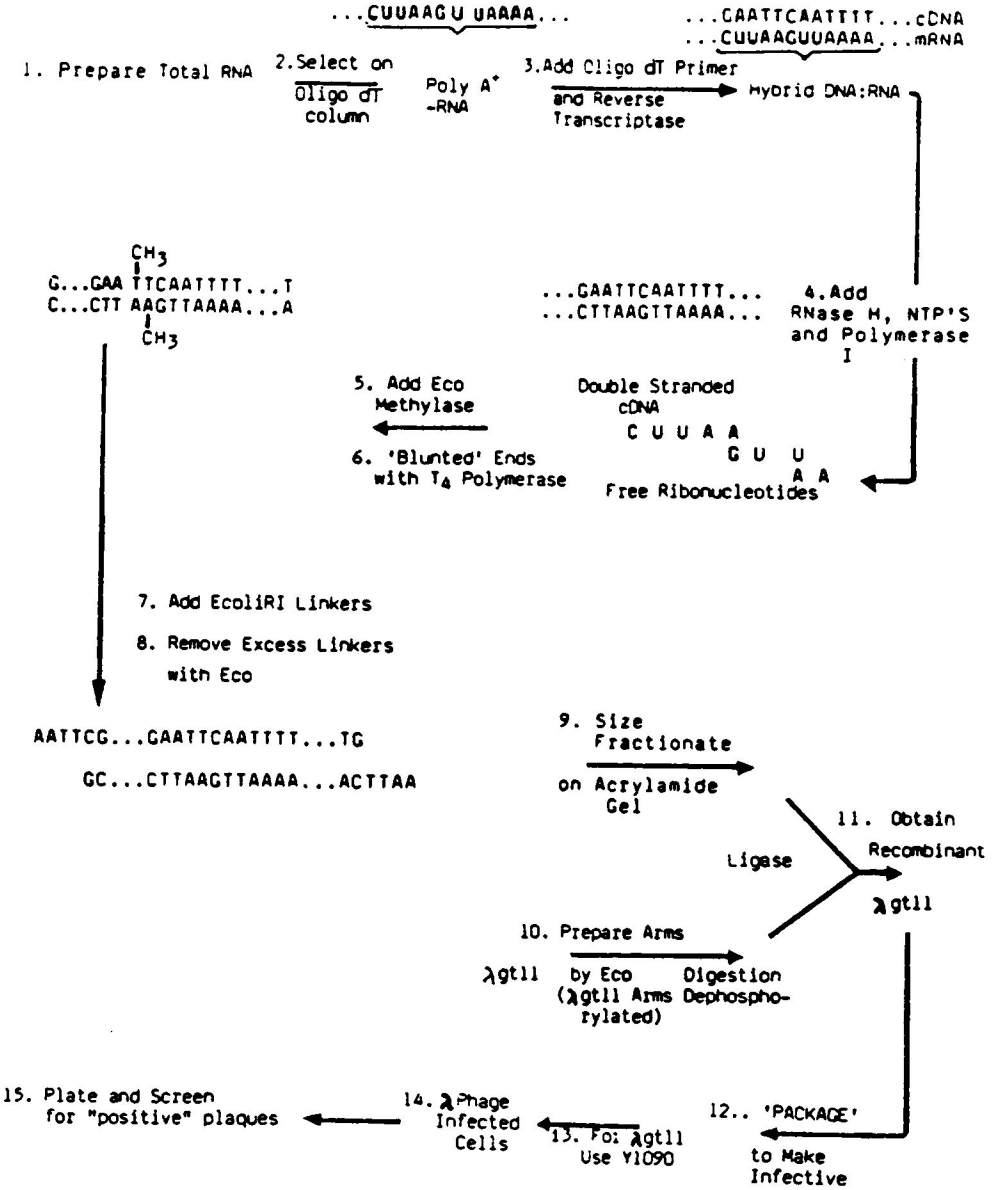
1 h. To the mixture was added 4.0 ul of 0.5 M EDTA, pH 8.0, and 100 ul of SS-phenol/chloroform (1:1 mix), mixed thoroughly and microcentrifuged for 2 min to separate the phases. The upper aqueous phase was transferred to a new microfuge tube, 100 ul chloroform were added and the suspension was mixed well and microcentrifuged for 2 min. The process was repeated before the addition of 100 ul of 4 M ammonium acetate to the upper aqueous phase. Six hundred microliters of 100% ethanol were added and the mixture frozen on dry ice for 30 min before microcentrifugation at 4°C for 10 min. The supernatant was decanted and the pellet resuspended in 50 ul of H_2O and 50 ul of 4 M ammonium acetate and 300 ul of 100% ethanol were added before freezing on dry ice for 30 min. The sample was microcentrifuged at 4°C for 10 min and the supernatant carefully decanted. To the pellet was added 150 ul of 80% ethanol (kept cold on ice). The sample was centrifuged for 2 min at 4°C, and the supernatant was carefully decanted, so as not to disturb the pellet. The pellet was dried under vacuum and resuspended in 25 ul of autoclaved H_2O in a 1.5 ml microcentrifuge tube.

EcoR1 methylation reaction, blunt end polishing and digestion to generate cloning ends were performed as recommended in Davis et al. (1986). The second strand recovery rate was determined by TCA precipitation of 2 ul of the sample and counting on a scintillation counter with toluene based cocktail. Figure 5 shows a schematic diagram for the construction of a Lambda gt11 total rat liver cDNA library.

Polysome Immunoadsorption for Specific UDPGT mRNA Purification.

The procedure described by Kraus and Rosenberg (1982) was employed here. Polysomes were prepared from the treated rats by standard methods (El-Dorry, 1986). Briefly, livers were homogenized in sufficient Tris buffer containing 50 mM Tris, pH 7.5, 25 mM NaCl, 5 mM $MgCl_2$, 0.25 M sucrose, 1 mg per ml Bentonite, 0.2 mg per ml heparin and 1 ug per ml cycloheximide to make a 15% (w/v) homogenate. The homogenate was collected and diluted in 0.1 vol of 10% (w/v) sodium deoxycholate, 10% (w/v) Triton X-100, before layering (31 ml) over a discontinous sucrose gradient containing 3 ml of 2.5 M sucrose and 1.5 ml of 2.0 M sucrose. Both sucrose solutions were prepared in a solution of 25 mM Tris pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, containing heparin (0.2 mg/ml) and cycloheximide (1 ug/ml). The sucrose gradient

Fig. 5. Schematic diagram of procedure for generating double-stranded cDNA species from mRNA, adding EcoR1 ends to the cDNA, and cloning into lambda gtll (Davis et al., 1986).


...CUUAAGU UAAAA...
...GAATTCAATTTT...cDNA
...CUUAAGUUAAAA...mRNA
1. Prepare Total RNA
2.Select on Oligo dT column
Poly A+ -RNA
3.Add Oligo dT Primer and Reverse Transcriptase
Hybrid DNA:RNA
CH3
G...GAA TTCAATTTT...T
C...CTT AAGTTAAAA...A
CH3
...GAATTCAATTTT...
...CTTAAGTTAAAA...
4.Add RNase H, NTP'S and Polymerase I
5. Add Eco Methylase
6. 'Blunted' Ends with T4 Polymerase
Double Stranded cDNA
C U U A A
G U U
A A
Free Ribonucleotides
7. Add EcoliRI Linkers
8. Remove Excess Linkers with Eco
AATTCG...GAATTCAATTTT...TG
GC...CTTAAGTTAAAA...ACTTAA
9. Size Fractionate on Acrylamide Gel
11. Obtain Recombinant λgt11
Ligase
10. Prepare Arms
λgt11 by Eco (λgt11 Arms Rylated)
Digestion Dephospho-
12.. 'PACKAGE' to Make Infective
13. For λgt11 Use Y1090
14. λ Phage Infected Cells
15. Plate and Screen for "positive" plaques

was then centrifuged for 17 h at 26,000 rpm. After centrifugation, the supernatant including the 2.0 M sucrose layers were aspirated, and the 2.5 M sucrose layers containing the polysome were added to 1 vol. of a standard polysome buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 0.1% Nonidet P-40 containing cycloheximide (1 ug/ml) and heparin (0.2 mg/ml). The polysome solution was dialyzed against 2 liters of the same buffer for 20 h, followed by a quick freeze in a dry ice/ethanol bath, and storage at -80°C until needed.

When needed, the polysome preparation was centrifuged for 10 min at 12,000 x g to remove aggregates and resuspended in the polysome buffer to a concentration of 15 A_{260} units/ml. Then the polysomes were reacted for 1 h at 4°C with specific rabbit anti-UDPGT IgG. Protein A sepharose was prepared, packed in a column that was washed with 1 M acetic acid and equilibrated with about 120 ml of the polysome buffer. The flow rate of the column was adjusted to 5-6 ml per h before the application of the polysome antibody mixture. The flow-through fractions were quick frozen and saved for immunopurification of other mRNA species with different antibodies. After application of the sample, the resin was removed from the column, thoroughly resuspended in 2 vol. of polysome buffer, and

repacked in the same column. The column was washed overnight with the purification buffer before elution of the ribosomal subunits and specific mRNA with the polysome buffer containing 25 mM Tris, 20 mM EDTA, pH 7.5 and heparin (0.2 mg/ml).

Sometimes, polysomal RNA was isolated by a simplified version of the above process as documented by El Dorry, 1986. In this procedure, rat liver was minced in 2.5 vol of 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 50 mM KCl (polysome buffer), containing 0.5 mg/ml heparin. The tissue was homogenizied with ten strokes using a teflon homogenizer, and centrifuged for 15 min at 1200 x g to remove unhomogenized cells, nuclear materials and mitochondria. The resulting supernatant was adjusted to 1% each of Triton X-100 and sodium deoxycholate, loaded at 2 ml/tube on 1.4 M sucrose in polysome buffer containing 0.5 mg/ml heparin and centrifuged at 45,000 rpm in a Beckman TY Ti-65 rotor for 150 min. The polysomal pellet was resuspended in polysome buffer containing 0.5 mg/ml heparin and 0.2 M NaCl. The final concentration of polysome was adjusted to 50 A_{260} units/ml and stored at -80°C in 500 ul aliquots until needed. The polysomes were sometimes subjected to <u>in vitro</u> translation as a check on the isolation process.

To isolate, total RNA, the polysomes were incubated at 4°C for at least 1 h with anti UDPGT IgG and eluted with 20 mM EDTA, pH 7.5, containing 0.5 mg/ml heparin.

Isolation of UDPGT Specific mRNA Following Polysome Immunoadsorption Using Oligo (dT) Chromatography

To isolate specific poly(A^+) RNA, the EDTA eluate was immediately diluted in 0.5 M LiCl and 0.5% sodium dodecyl sulfate (SDS) and applied to a 2 ml column of oligo (dT)-cellulose. The column was washed with 60 ml of the binding buffer (1 mM EDTA, 0.5 M LiCl, 0.5% SDS and 10 mM Tris-HCl, pH 7.5), and the bound RNA eluted with the elution buffer (1 mM EDTA, and 10 mM Tris-HCl, pH 7.5) and then precipitated at -20°C with 2 vol. of ethanol and 0.05 vol of 4 M NaCl in the presence of 60 ug of rat liver tRNA (Boehringer Mannheim).

In Vitro Translation of the mRNAs and Immunoprecipitation of their Specific UDPGT

The specific UDPGT poly (A^+) RNA was translated in vitro using a nuclease-treated rabbit reticulocyte system prepared as described by Pelham and Jackson (1976) or using the reticulocyte translation kit according to the instructions of the supplier (BRL Inc.).

Reticulocyte lysate was obtained from rabbits that had been made anemic by subcutaneous injection of 1.2% acetylphenylhydrazine for 5 days (Krajewska et al., 1986), and the lysate resuspended in 25 uM hemin and 1 mM $CaCl_2$, and then incubated with micrococcal nuclease (30 ug/ml) for 15 min at 20°C. After cooling to 4°C, creatine phosphokinase and EGTA (pH 7.0) were added to a final concentration of 50 ug/ml and 2 mM, respectively, and the lysates stored at -80°C. The cell free reaction mixture contained 40% (v/v) of freshly thawed lysate, 2-4 ug/ml poly (A^+) RNA, 0.025 mM of each of the 19 unlabelled amino acid, 200 uCi/ml of [^{35}S]-methionine (New England Nuclear, 887-1058 Ci/mMole), 80 mM KCl, 1 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 8 mM creatine phosphate and 20 mM HEPES, pH 7.6. The mixture was incubated for 90 min at 30°C, followed by a cold 25% TCA precipitation for 10 min at 4°C. The precipitated protein was collected on glass fiber discs (Whatman GF/C) which were washed with 8% TCA. Prior to determination of radioactivity, filters were solubilized in protosol tissue solubilizer (Amersham) and counted in aqueous scintillation cocktail (Amersham). Total incorporation of labelled methionine was determined by incubating an aliquot of the reaction mixture (2 ul) with 0.5 M NaOH, 0.05% methionine, 0.7% H_2O_2 for 15 min at 37°C.

For immunoprecipitation of the in vitro translated proteins, the sample was diluted to 0.2 ml with buffer A containing 150 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 2% methionine, 0.2% SDS, 0.5% BSA, 0.02% sodium azide and 10 mM sodium phosphate, pH 7.5 and 3 ul of normal rabbit serum added (pre-immune serum). After incubation of the mixture at room temperature for 30 min, 30 ul of 10% (w/v) fixed Staphylococcus aureus cells were added and incubated with the mixture for 30 min on ice before centrifugation at 10,000 x g for 5 min (Fagan et al, 1983). The pellet was saved at -80°C and to the supernatant was added 3 ul of rabbit-anti-rat UDPGT immunoglobulins for an additional 30 min incubation at room temperature before transferring to 4°C to incubate overnight. The S. aureus step and washing was repeated for a second time (Jackson et al., 1986). To recover the immunoprecipitates, the mixture was layered over a 1 M sucrose cushion (1 ml) in buffer A and centrifuged at 10,000 x g for 10 min. The pellet was first washed with Buffer A and then 150 mM NaCl in 10 mM sodium phosphate, pH 7.2. The radioactivity was determined by liquid scintillation spectrometry after solubilizing the pellet in protosol tissue solubilizers.

To assay for the interrelationships of the p-nitrophenol UDPGT forms in cold immunoprecipitated proteins,

purified UDPGT from different treatments were mixed with anti UDPGT sera in Buffer A, and incubated in ice for 2 h. Also, the same sera was used to immunoprecipitate the *in vitro* translation products labelled with [^{35}S]-methionine. Furthermore, SDS/polyacrylamide gel electrophoresis (10% resolving and 3% stacking) was performed on the [^{35}S]-labelled translation products, then fixed, dried and the gels autoradiographed. Before the SDS/PAGE, isolated antigens were released from the *S. aureus* cells by heating at 100°C for 5 min in sample buffer containing SDS and 2-mercaptoethanol (Jackson et al., 1986), followed by centrifugation at 40,000 rpm for 60 min at 4°C to pellet the ribosomes.

Autoradiography of In Vitro Translation Proteins

[^{35}S]-methionine labelled proteins were electrophoresed on 10% SDS/PAGE as described by Laemlli, 1970. The gels were dried using Bio-Rad drying equipment and then exposed at -70°C for two weeks using Kodak XAR-5 film. The cassettes were removed from -70°C and allowed to thaw before opening. Later on, the films were immersed in the developer for 5 min, rinsed in water for 2 min and then immersed in the fixative for 5 min. All films were allowed to air dry

for about 30 min. Molecular sizes were estimated by comparison to known molecular weight standards.

Trypsin Digestion of Purified UDPGT from 3-MC and BHA Treated Rats

P-nitrophenol UDPGT from BHA and 3-MC treatments obtained by affinity chromatography were subjected to limited protease digestion using trypsin. Results from the two treatments were analyzed on HPLC for differences and similarities. The peptides generated by proteolysis were analyzed in 15% acrylamide gels (30.0% by weight acrylamide: 0.8% Bisacrylamide) as originally described by Cleveland et al (1977) and modified by Tukey et al. (1981). The protein bands were visualized by placing the slab gel in 4.0 M sodium acetate.

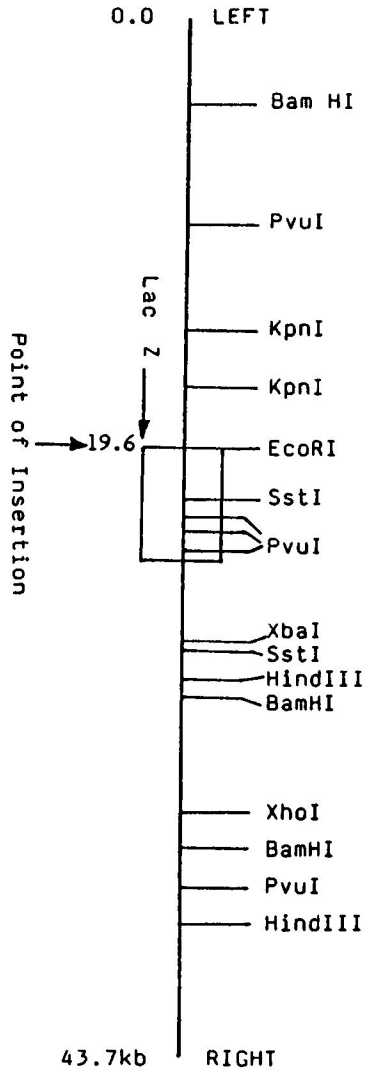
Procedure for Screening Lambda gt11 Recombinants Using Polyclonal P-nitrophenol UDPGT Antibody

E. coli Y1090 (Clontech Lab, Palo Alto, CA) was plated on 85 mm LB plates (10g tryptone, 5g yeast extract, 5g sodium chloride per liter plus 1.5% agar) containing 50 ug/ml ampicillin as instructed in the Clontech manual. After 18 h incubation, an isolated single colony was picked and grown to saturation overnight in LB broth containing 0.2% maltose (sterilized by autoclaving or filteration in 0.2 um millipore) and 50 ug/ml ampicillin with shaking. Bacterial

cells (0.5 ml) from the overnight culture were incubated with 10 ul of the appropriately diluted Lambda gt11 recombinant bacteriophage (Clontech Lab) in the diluent buffer (10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and 0.1 mM EDTA) for 15 min at 37°C for the phages to attach to the bacterial host. Following incubation at 52°C, 7.5 ml of soft agar (LB broth plus 0.75% agar) was added to the culture tube, mixed gently and poured onto a 150 mm LB plate. The plates were incubated at 42°C for 3-4 h, after which they were removed to a 32°C or 37°C incubator and overlaid with dry nitrocellulose filter presoaked in 10 mM IPTG, and allowed to air dry for 10 min, before overnight incubation.

To check the cDNA library for presence of recombinants, to the 0.5 ml bacterial cell culture and 10 ul of the bacteriophage mixture mentioned above, were added 3.0 ul X-gal (10% in DMF) and 3 ul IPTG (100 mM/ml) and incubated at 37°C for 15 min. Then the soft agar was added, the plate poured, followed by incubation at 42°C as indicated earlier (Davies et al, 1986). Here, blue plaques represent parental Lambda gt11 with functional beta-galactosidase (lacZ) gene while clear plaques represent recombinants with defective lacZ gene. The recombinant is a hybrid of the cDNA insert and portions of the lacZ gene (Fig. 6).

Fig. 6. Illustrative diagram of lambda gtll cloning vector showing restriction sites and point of cDNA insertion.


0.0
LEFT
Bam HI
PvuI
Lac Z
KpnI
KpnI
Point of Insertion
19.6
EcoRI
SstI
PvuI
XbaI
SstI
HindIII
BamHI
XhoI
BamHI
PvuI
HindIII
43.7kb
RIGHT

To screen the filters for p-nitrophenol UDPGT specific clones, the filters were removed from the 37°C incubator, transferred to a fresh 150 mm petri dish with the side of the filter that was in contact with the soft agar up, and rinsed in TTBS (50 mM Tris (pH 7.9), 150 mM NaCl plus 0.05% Tween 20) for 20 min. Later, the filter was incubated in 10 ml TTBS plus 20% fetal calf serum for 30 min at room temperature, after which it was removed and rinsed in TTBS. In binding the primary antibody, the filter was incubated in 10 ml TTBS plus 1:20 dilution (1 mg/ml) of the rabbit anti rat p-nitrophenol UDPGT IgG at room temperature. The filter was then washed in three changes in TTBS, 5 min each change. For the bound primary antibody to be detected, goat anti-rabbit IgG conjugated with horse-radish peroxidase was employed as the secondary antibody. Here, the filter was transferred to 10 ml TTBS containing affinity purified secondary antibody that was diluted at 8 ul IgG/10 ml TTBS. The reaction mixture was incubated at room temperature with gentle agitation. After 1 h of incubation, the filter was washed in three changes of TBS (no Tween 20), 5 min each change. Finally, the nitrocellulose filter was incubated in 10 ml peroxidase substrate solution (2 ml of 4-chloro-1-napthol (3 mg/ml) in methanol and 10 ml TBS plus 0.01 M

imidazole after which was added 5 ul of 30% hydrogen peroxide) for approximately 30 min. The peroxidase solution was used immediately after mixing. Before storing the filter in the dark to avoid bleaching of the positive stains, it was washed in three changes of distilled water and air dried.

Prior to usage, the primary antibody (3 ml) was preadsorbed overnight against a lysate (100 ul) of E. coli Y1090 cells as recommended by French et al. (1986). The E. coli lysate was prepared by resuspending Y1090 cells from a 100 ml overnight culture in 12 ml distilled water, and heated in a boiling water bath for 5 min before freezing at -20°C in 500 ul aliquots. Usually, the primary antibody - bacterial lysate mixture (after overnight incubation at 4°C with rotation) was centrifuged at 4000 x g for 10 min to recover the preadsorbed antibody to be used for immunoscreening.

Affinity Purification of Antibodies Recognizing Cloned Protein (Antibody Select)

Antibodies from polyclonal antiserum were affinity purified as recommended by Barlow et al. (1987) with a few modifications. Lambda gt11 recombinant expressing UDPGT were grown for 35 h at 42°C, and the plaque proteins lifted onto 10 mM IPTG impregnated nitrocellulose filters overnight at 37°C. Before lifting the filters, the plates

were stored at 4°C for at least 1 h. The filters were washed with TTBS three times for 5 min each. They were then blocked with 3% BSA for 30 min at room temperature. After washing three times with TTBS they were incubated with a 1:20 dilution of rabbit anti-UDPGT serum for 4 h at room temperature with shaking. Antibodies that were unbound were removed by washing three times in TTBS. The last wash was in physiological saline. The bound antibodies were eluted with 5 ml per 150 mm petri dish of 0.15 M glycine hydrochloride, pH 2.3 for 15 min. Here, the agar plates utilized in growing the bacteria were used after the agar had been removed and the plate treated with glycine-NaCl pH 2.8 buffer, to prevent antibody from adhering to the plates. The eluate was neutralized by adding 8 mg Tris/ml eluent and stored at -20°C in 500 ul aliquots. Eluted serum was diluted 1:10 in Western blot assays.

Microsomal proteins (1.0 mg) from different treatments were fractionated on 7.5% SDS/PAGE with 3% stacking for 10 h at 75 mA and constant voltage, and electrophoretically transferred to nitrocellulose membranes at 150 mA overnight in 25 mM Tris-192 mM glycine-20% methanol. The membranes were processed as described in the Western blot assay technique.

Preparation of Phage DNA

DNA from the positive recombinant clones was prepared from 150 mm petri-dishes that were plaqued out. Each plaqued out plate received 7.5 ml of SM buffer and was incubated for 2 h at room temperature with shaking (Maniatis et al., 1982). To enhance the migration of the phages out of the soft agar, a drop of chloroform was added to the harvested supernatant and incubated at 4°C for 4 h. The supernatant was treated with 1 ug/ml DNase 1 for 30 min at 37°C. An equal volume of a solution containing 20% (w/v) polyethylene glycol-6000 (PEG-6000) and 2 M NaCl in SM was added and incubated for 1 h at 0°C (ice water). The precipitated phage particles were recovered by centrifugation at 10,000 x g for 20 min at 4°C. The supernatant was removed and the air-dried pellet resuspended in 0.5 ml of SM by vortexing. To remove debris, the sample was centrifuged at 8000 x g for 2 min at 4°C, and the supernatant transferred to an eppendorf tube, after which 5 ul of 10% SDS and 5 ul of 0.5 M EDTA (pH 8.0) were added before heating at 68°C for 15 min. The sample was extracted once with SS-phenol, once with phenol/chloroform (1:1), and once with chloroform. In each case the top aqueous phase was transferred to a fresh eppendorf tube. To the final aqueous phase was added an

equal volume of isopropanol, and it was stored at -70°C for 30 min, then thawed and centrifuged in a microfuge for 15 min at 4°C. The pellet was washed with 70% ethanol, dried by vacuum and resuspended in 50 ul of TE (10 mM Tris-HCl, pH 8,0.1 mM EDTA, pH 8.0) buffer, and stored at -20°C until needed.

CHAPTER IV

EXPERIMENTAL RESULTS

Table 1 shows the effect of the treatments on the animal weight, liver weight and a ratio of the liver weight and body weight. Administration of BHA, 3-MC or BHA + 3-MC to rats resulted in appreciable changes in the animals. BHA + 3-MC treatment showed an additive effect in liver weight and body weight compared to either BHA or 3-MC alone, however, measurable increase in body weight was observed in all the treatments in relation to the untreated rats. No detectable difference in liver weight was observed between the BHA and 3-MC treatments. Hepatomegaly was evident following all treatments; this was more pronounced in the BHA + 3-MC treated rats. In addition, hyperlipidemia was observed in the treated rats compared to the control, which was more pronounced in the BHA + 3-MC simultaneous induction.

The liver versus body weight ratio showed no difference in the single treatments of BHA and 3-MC compared to the control, or between the two treatments. However, the result for the combined treatment was significantly different from that of the single treatments or the control.

Table 1. Effects of Inducer Agents on Rat and Liver Weights, Liver/Body Weight Ratio

Treatment	Animal Weight at Sacrifice (g)	Liver Weight (g)	Liver/Body Weight X 100
Control	262.5 ± 18.8 (06.0)*	7.50	2.86
BHA	246.2 ± 14.1 (22.0)*	7.44	3.02
3MC	262.8 ± 33.1 (25.0)*	8.40	3.20
BHA + 3MC	260.2 ± 12.4 (59.0)*	12.20	4.69

*Numbers in parenthesis represent change in body weight by the time of sacrifice.

Values for animal weight and liver are the means for 10 animals.

P-nitrophenol UDPGT Purification by Ion-Exchange and Affinity Chromatography

Solubilized microsomes from untreated control, 3-MC, BHA and BHA + 3-MC treated rats were fractioned on DEAE-cellulose (Fig. 7) to partially purify the enzyme. Except for the control group, four major protein peaks were obtained from each treatment, and labelled A, B, C, and D. Peak B was the missing peak in the control group. Since Peak A was eluted before the addition of the salt gradient, the protein(s) in this peak were considered cationic, which include all hepatic proteins that cannot bind to DEAE-cellulose. The pooled and concentrated peak A fractions had a brown color which is indicative of the presence of heme containing proteins, possibly cytochrome.

Peaks B-D were obtained from 0-500 mM KCl salt gradient elution. These peaks in all treatments, spread from low to high salt concentrations, and their proteins were referred to as anionic proteins. The absence of B in the control group seems to indicate that the low-salt forms are inducer specific. The BHA and 3-MC + BHA peaks were not as well resolved on DEAE-cellulose as the control and 3-MC peaks.

Following the DEAE-cellulose chromatography, peak A and peaks B-D were pooled separately for each treatment, and were identified as non-salt and salt peaks,

Fig. 7. DEAE-cellulose ion-exchange chromatography. Peak "A" is non-salt, and Peaks B-D are "salt" fractions.

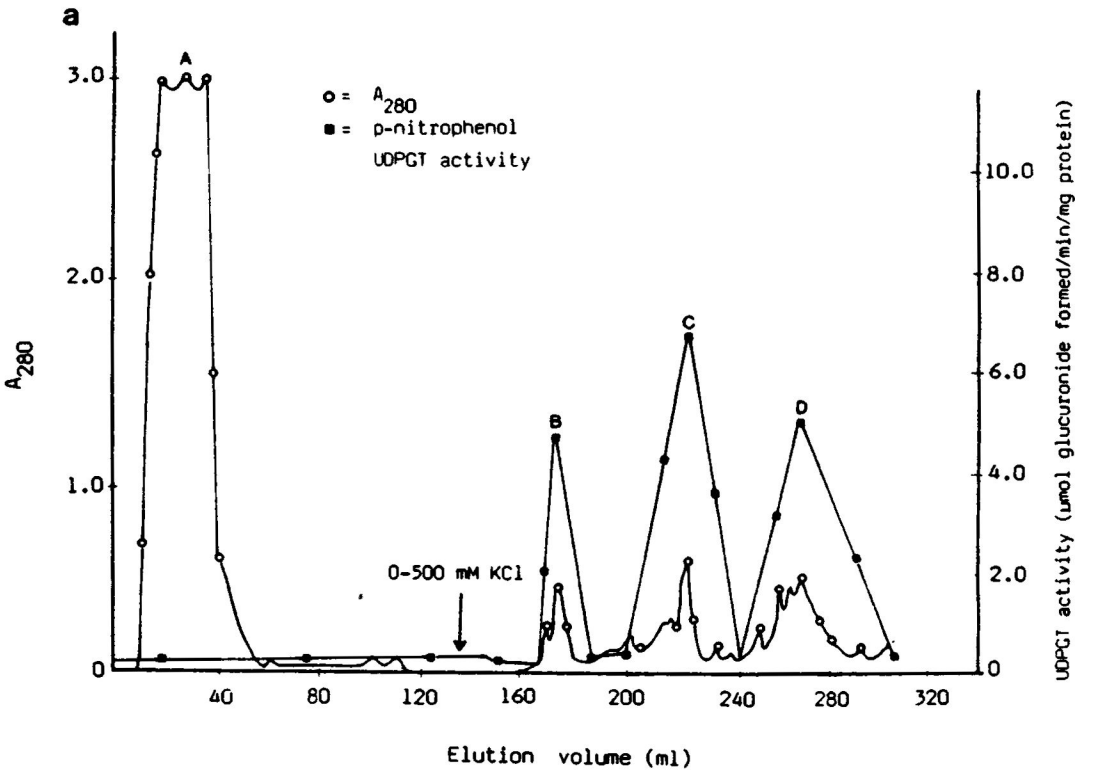
a. Prototype profile showing total protein and UDPGT activity distribution.

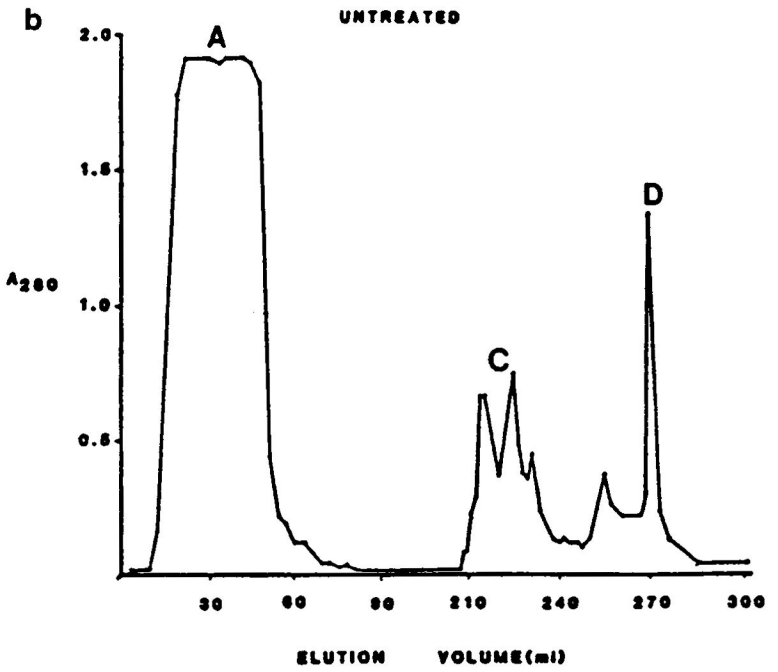
b. Untreated profile

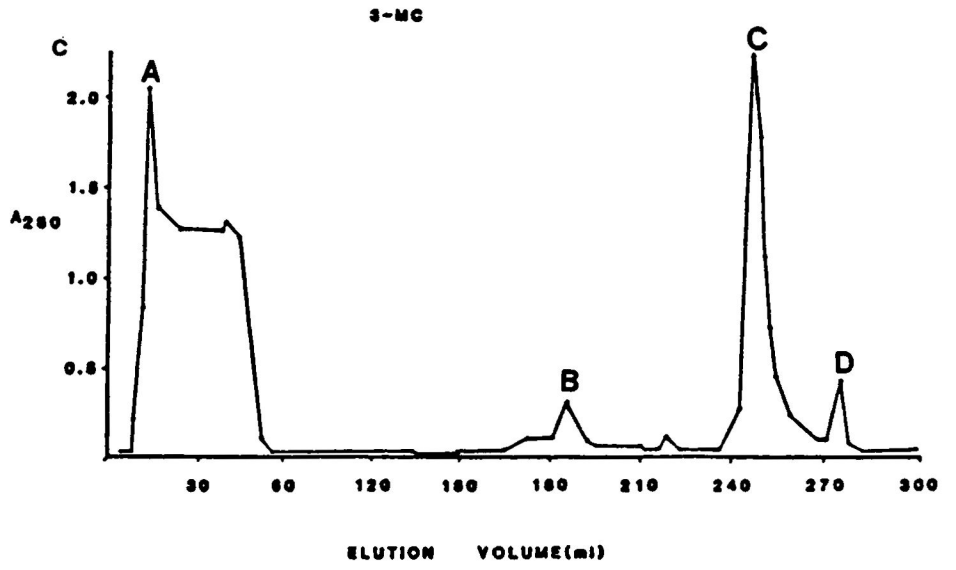
c. 3-MC profile

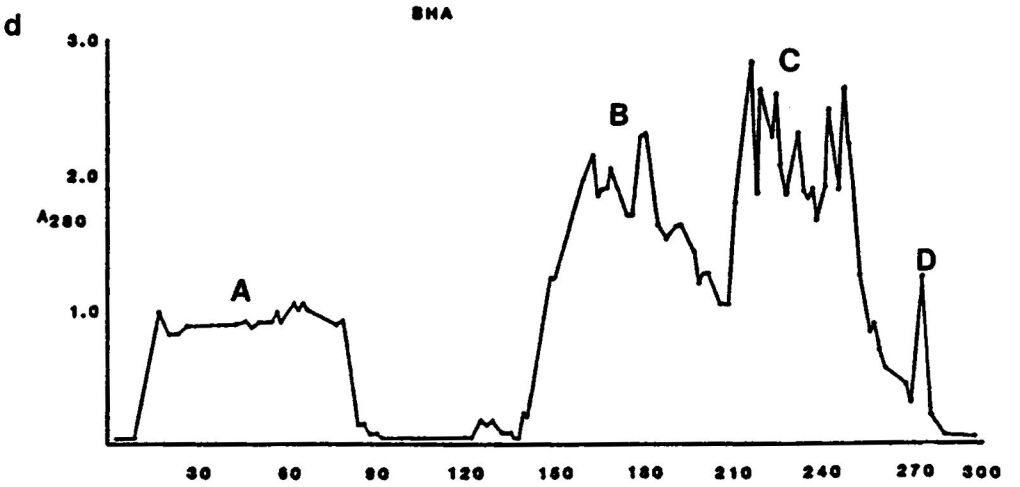
d. BHA profile

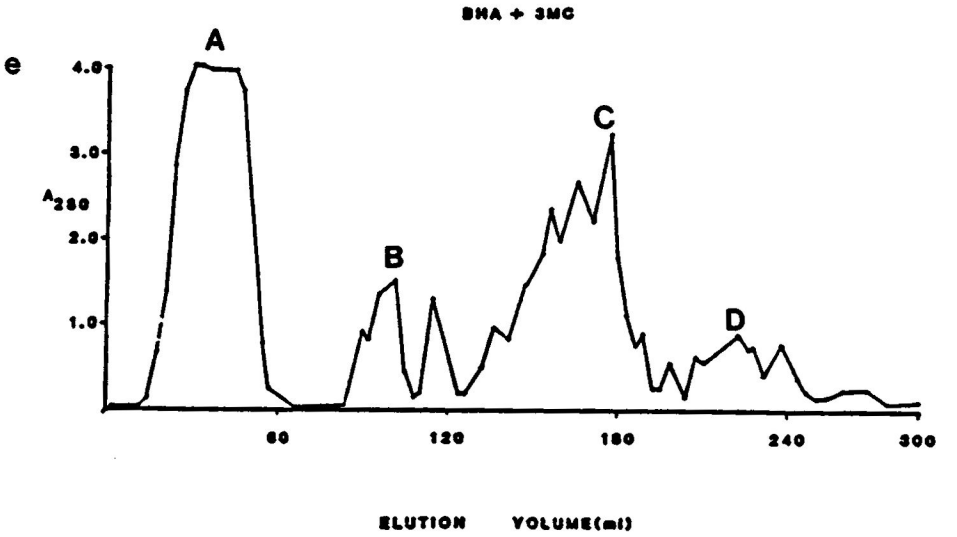
e. BHA + 3-MC profile


a
A
B
C
D
o = A_{280}
■ = p-nitrophenol UDPGT activity
0-500 mM KCl
A_{280}
3.0
2.0
1.0
0
40
80
120
160
200
240
280
320
10.0
8.0
6.0
4.0
2.0
0
UDPGT activity (µmol glucuronide formed/min/mg protein)
Elution volume (ml)


b
UNTREATED
A
C
D
2.0
1.5
1.0
0.5
A280
30
60
90
210
240
270
300
ELUTION VOLUME(ml)


3-MC
c
A
B
C
D
2.0
1.5
1.0
0.5
A280
30
60
120
150
180
210
240
270
300
ELUTION VOLUME(ml)


d
BHA
3.0
2.0
A_{280}
1.0
A
B
C
D
30
60
90
120
150
180
210
240
270
300
ELUTION VOLUME(ml)


BHA + 3MC
e
A
4.0
3.0
A_{280}
2.0
1.0
B
C
D
60
120
180
240
300
ELUTION VOLUME(ml)

respectively. These fractions were individually applied to a UDPGA-agarose affinity column and eluted with 5 mM UDPGA in 50 mM KCl. Figure 8 shows the elution profiles of control, 3-MC, BHA and BHA + 3-MC salt fractions on affinity chromatography. Irrespective of treatment, two major peaks were obtained. The first peak eluted before the addition of 5 mM UDPGA, and the second peak eluted after UDPGA addition. Quantitative differences were obtained in the amount of protein eluting before and after UDPGA addition among the treatments. When the non-salt peaks were subjected to UDPGA-agarose affinity fractionation, two major peaks were obtained as in the fractionation of the salt peaks (Fig. 9).

Measurement of P-nitrophenol UDPGT Activity

Table 2 shows a typical p-nitrophenol UDPGT activity profile of the peaks obtained during purification. Although the data presented here was obtained from BHA + 3-MC treated animals, similar profiles were observed in the other treatments. Based on the results that were obtained, there was minimal p-nitrophenol UDPGT activity in the non-salt fractions of the DEAE-cellulose when the solubilized microsomes were subjected to this ion-exchange fractionation.

Fig. 8. UDPGA-agarose affinity chromatography of the pooled "Salt" peaks. Peak 1 is the flow through and peak 2 is the 5 mM UDPGA eluate.

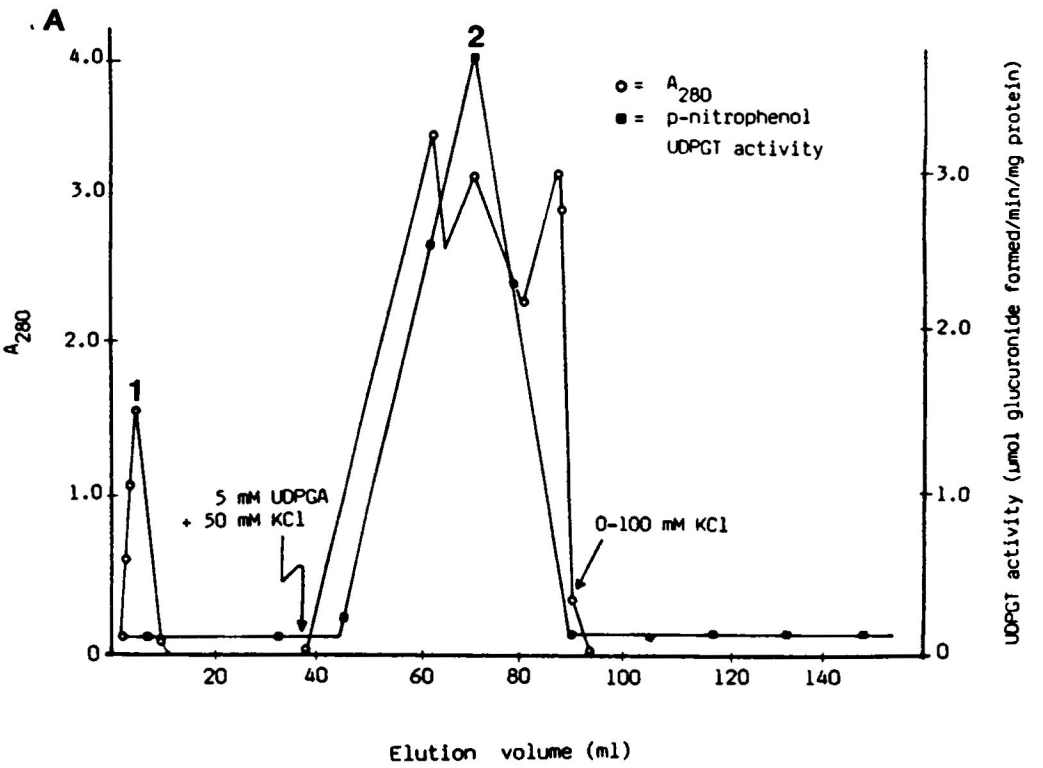
A. Prototype profile showing protein and activity distribution.

B. Untreated profile

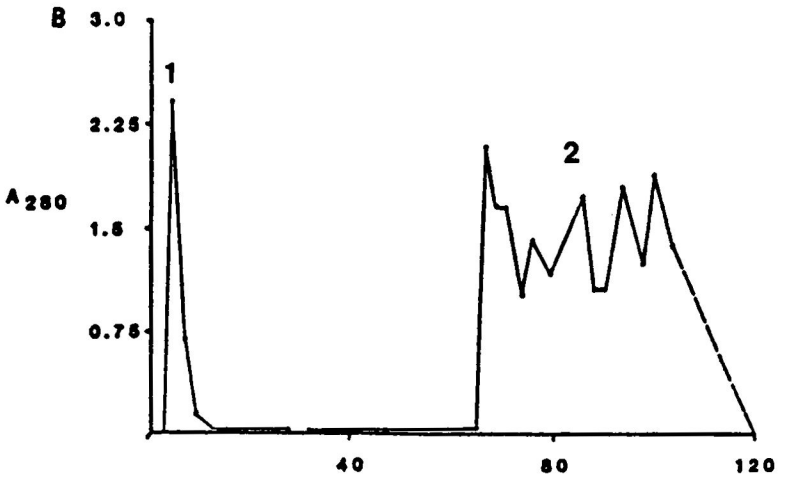
C. 3-MC profile

D. BHA profile

E. BHA + 3-MC profile

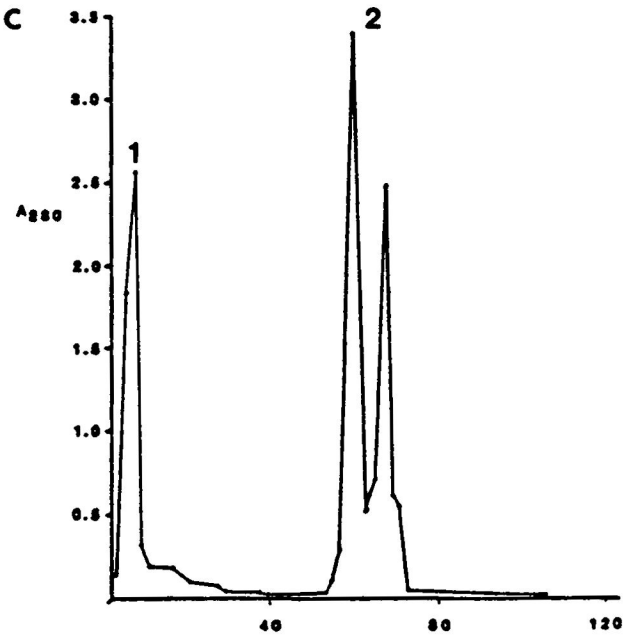

A
4.0
3.0
2.0
1.0
0
A_{280}
1
2
o = A_{280}
■ = p-nitrophenol UDPGT activity
5 mM UDPGA
+ 50 mM KCl
0-100 mM KCl
20
40
60
80
100
120
140
Elution volume (ml)
UDPGT activity (µmol glucuronide formed/min/mg protein)

UNTREATED

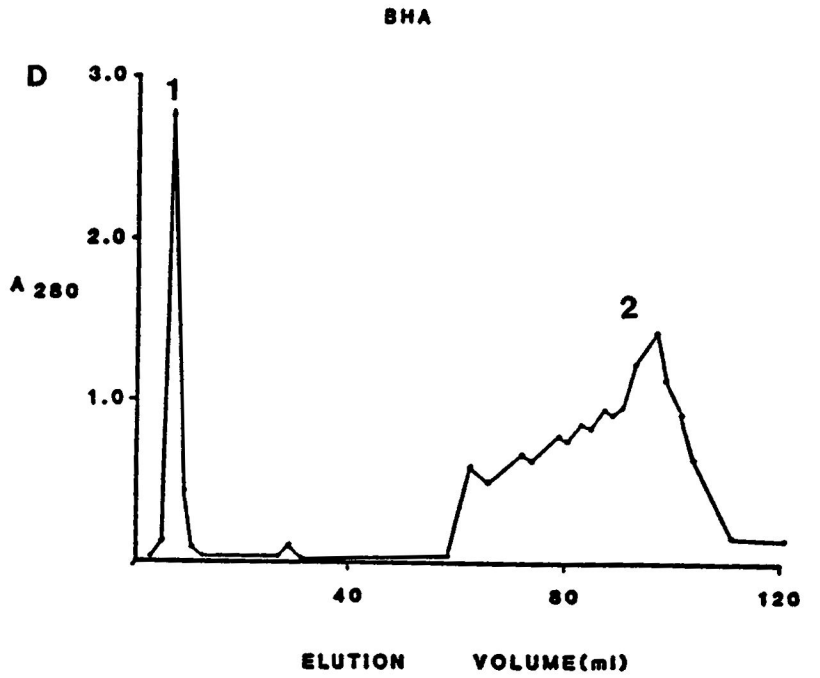


ELUTION VOLUME(ml)

SMC



ELUTION VOLUME(ml)


BHA
D
3.0
2.0
1.0
A 280
1
2
40
80
120
ELUTION VOLUME(ml)

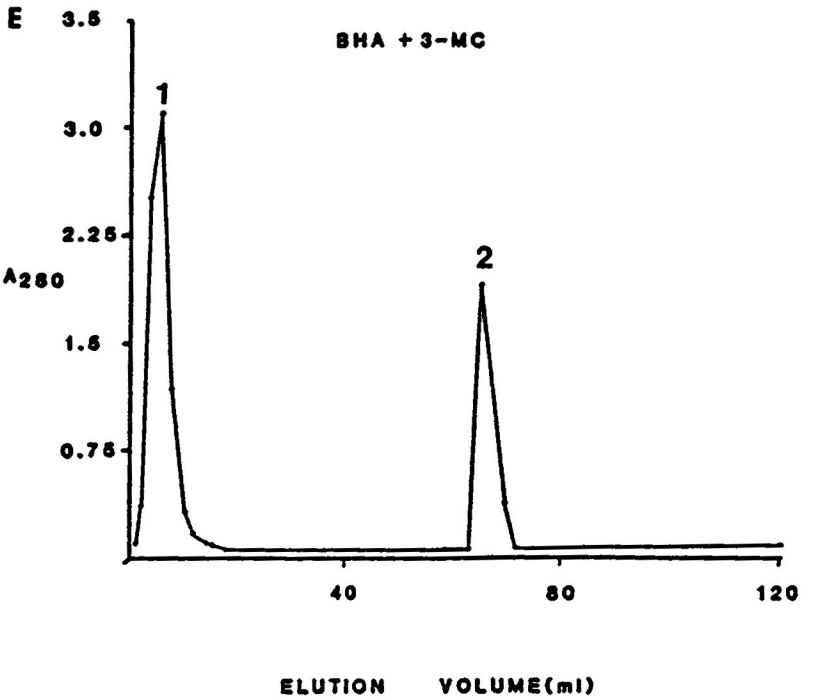

E
3.5
BHA + 3-MC
3.0
2.25
1.5
0.75
A280
1
2
40
80
120
ELUTION VOLUME(ml)

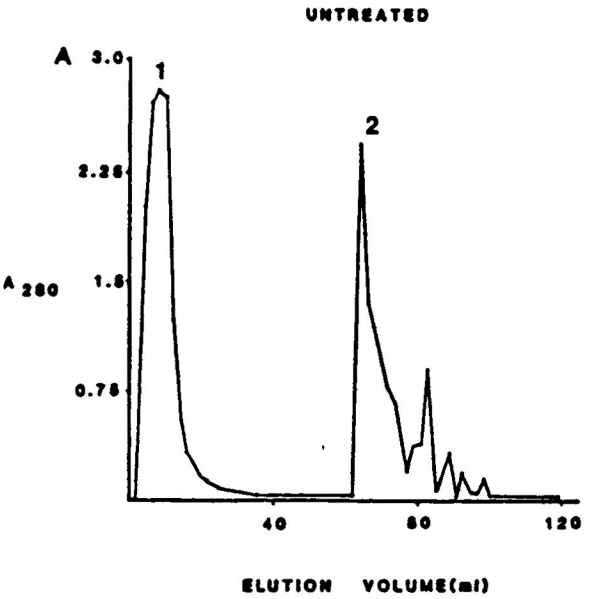
Fig. 9. UDPGA-agarose affinity chromatography of the "non-salt" peaks from the DEAE-cellulose separation.

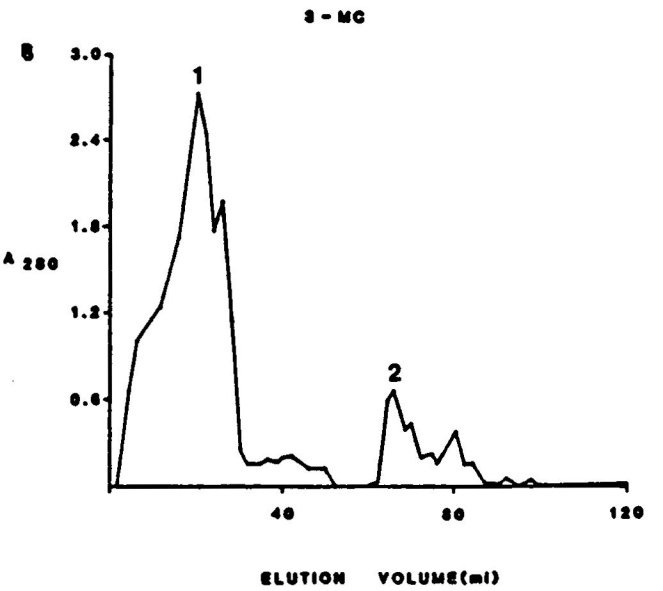
A. Untreated

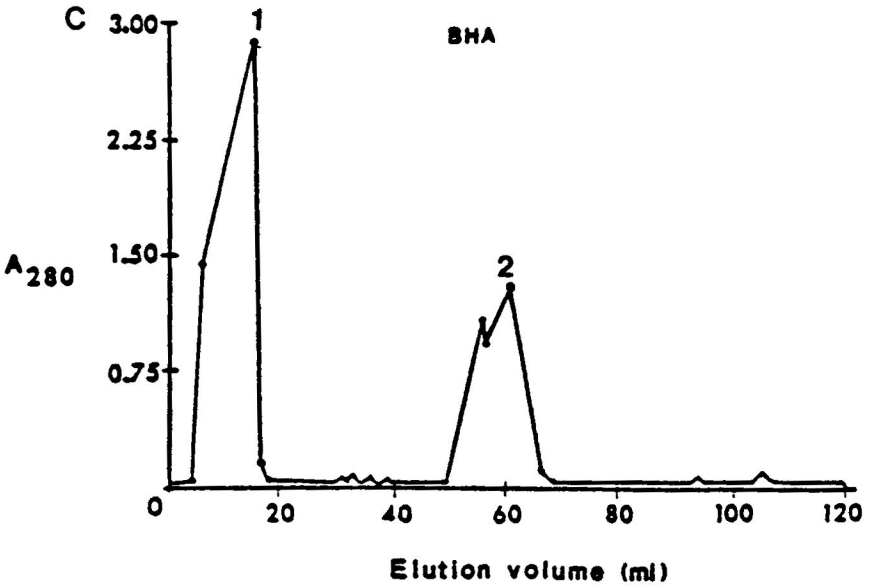
B. 3-MC

C. BHA

D. BHA + 3-MC


UNTREATED
A
3.0
2.25
1.5
0.75
A 280
1
2
40
80
120
ELUTION VOLUME(ml)


3-MC
B
3.0
2.4
1.8
1.2
0.6
A 280
1
2
40
80
120
ELUTION VOLUME(ml)


C
3.00
2.25
1.50
0.75
0
A_{280}
1
2
BHA
20
40
60
80
100
120
Elution volume (ml)

BHA + 3-MC

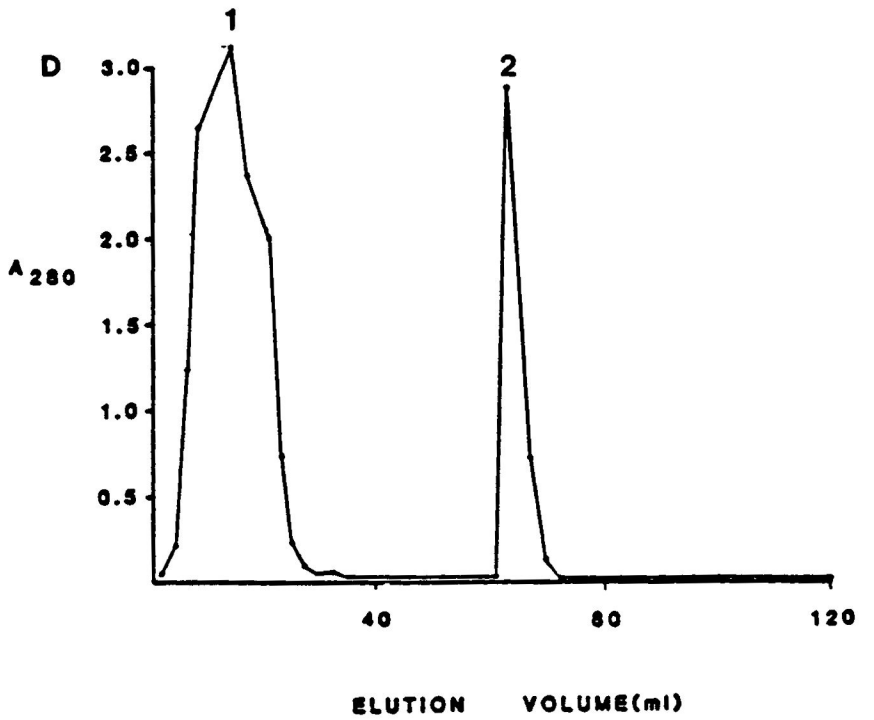

D
3.0
2.5
2.0
1.5
1.0
0.5
A_{280}
1
2
40
80
120
ELUTION VOLUME(ml)

Table 2. P-nitrophenol UDPGT Activity Profile.

Purification step		Apparent Sp. Activity (n=3) (umole Pnp/mg Protein/min)	Concentration mg/ml
Solubilized Microsome		1.369	1.84
DEAE-Cellulose Chromatography (0-500 mM KCl)	Peak A	0.129	1.00
	Peak B	241.369	0.01
	Peak C	36.549	0.10
	Peak D	128.268	0.02
UDPGA-Agarose Affinity Chromatography (DEAE-Peaks B-D)	Flow Through	0.580	0.202
	5 mM UDPGA Elution	480.570	0.078

Most of the p-nitrophenol UDPGT activity was detected in the intermediate and high salt fractions following 0-500 mM KCl elution. Although peaks B and D had less protein compared to A and C, there was more p-nitrophenol UDPGT activity in the former.

When the pooled salt fractions were subjected to UDPGA-agarose affinity fractionation, minimal p-nitrophenol UDPGT activity was detected in the flow-through. However, when the column was eluted with 5 mM UDPGA in 50 mM KCl, about 85% of the activity from the DEAE-cellulose fractions was recovered. Figure 10 shows a flow diagram of the purification scheme and activity distribution.

SDS/Polyacrylamide Gel Electrophoresis and Molecular Weight Estimation

Following the ion-exchange and affinity chromatographies, the proteins were subjected to a 10% SDS/PAGE analysis to determine the molecular weight of the purified UDPGT. The ion-exchange non-salt eluates showed two bands, at estimated molecular weights of 52 and 54 kilodaltons. Neither of these bands, which were visualized by silver staining (Fig. 11) were within the documented molecular weight range of rat liver p-nitrophenol UDPGT (Falany et al., 1986). Rat liver has been known to have other forms

Fig. 10. A flow diagram of the procedures used for the separation and purification of p-nitrophenol UDP-glucuronyltransferase.

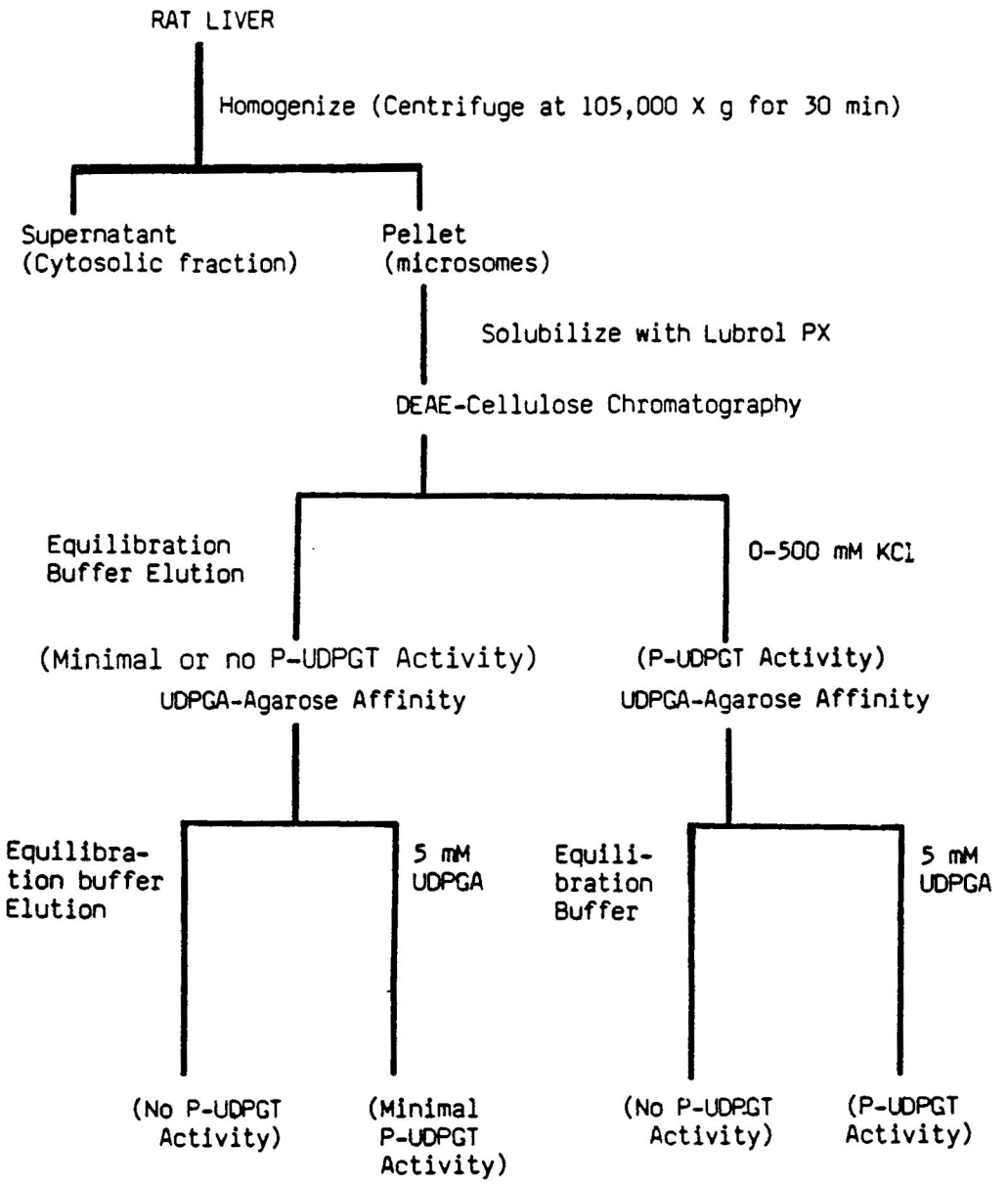

RAT LIVER
Homogenize (Centrifuge at 105,000 X g for 30 min)
Supernatant
(Cytosolic fraction)
Pellet
(microsomes)
Solubilize with Lubrol PX
DEAE-Cellulose Chromatography
Equilibration
Buffer Elution
0-500 mM KCl
(Minimal or no P-UDPGT Activity)
(P-UDPGT Activity)
UDPGA-Agarose Affinity
UDPGA-Agarose Affinity
Equilibra-
tion buffer
Elution
5 mM
UDPGA
Equili-
bration
Buffer
5 mM
UDPGA
(No P-UDPGT
Activity)
(Minimal
P-UDPGT
Activity)
(No P-UDPGT
Activity)
(P-UDPGT
Activity)

Fig. 11. Analysis of the "non-salt" and hydroxyapatite fractions after affinity purification. Proteins from different treatments were subjected to 10% SDS/PAGE as follows:

Lane 1. BHA, affinity purified

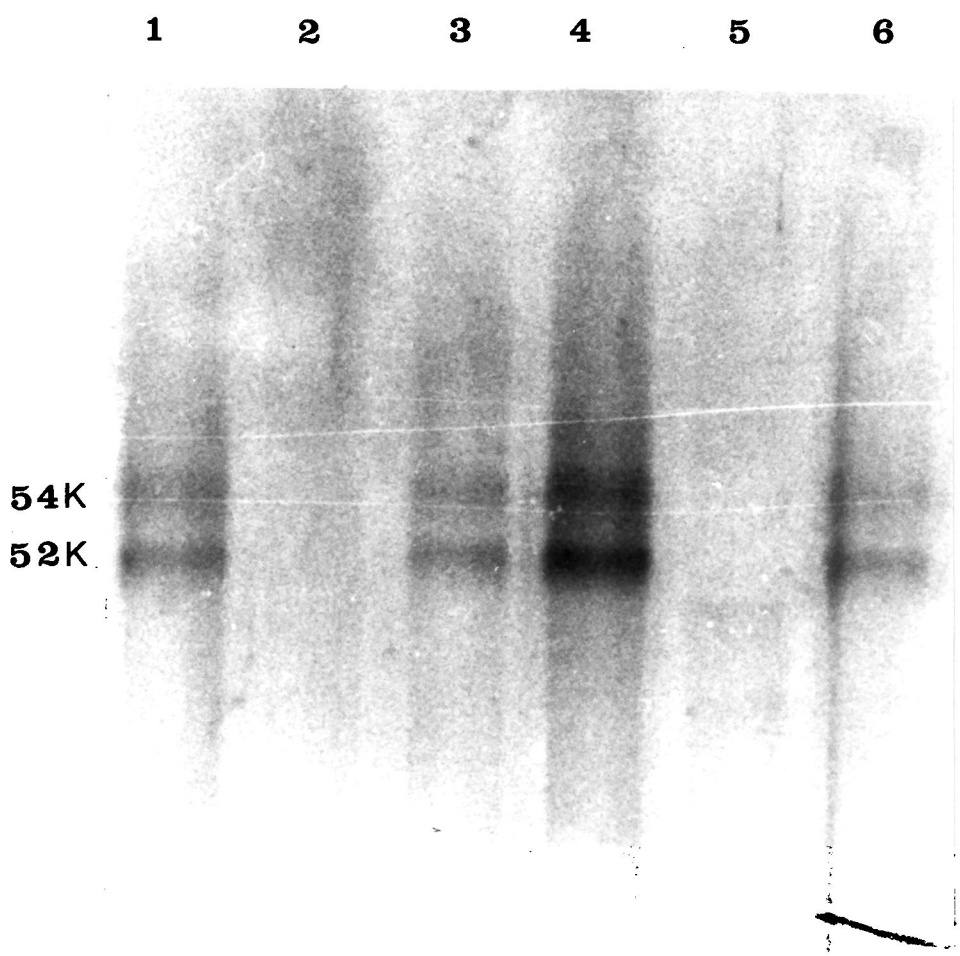
Lane 2. BHA, affinity flow through

Lane 3. BHA + 3-MC affinity purified

Lane 4. BHA + 3-MC, Hydroxyaptite purified

Lane 5. BHA + 3-MC, affinity flow through

Lane 6. 3-MC, affinity purified


1
2
3
4
5
6
54K
52K

of UDPGT (Tukey et al., 1982). They elute without salt, and migrate on SDS/PAGE at about 50-54k. Irrespective of the origin and nature of these proteins, they were observed in all the treatments. The affinity flow through samples had protein bands that migrated a little slower than the two predominant bands that could hardly be visualized on the gel. Here, we classified these as p-nitrophenol UDPGT or a related protein that contaminated the non-salt fractions during ion-exchange chromatography. Although the amount is relatively small, they may be responsible for the residual p-nitrophenol UDPGT activity detected in these fractions in the enzyme assays.

Following the electrophoretic analysis of the non-salt fractions, the salt samples were analyzed simultaneously with the non-salts for comparative purposes. The salt fractions had a major band whose electrophoretic mobility was less than that of the major band observed in non-salt fractions (Fig 12). In this analysis, the 5.0 ug/ml of protein used was not enough to visualize bands on the coomassie-blue stained gels. However, a minor protein band that migrated slower than the major band in the salt fractions was observed. This band was more distinct in the affinity purified samples.

Fig. 12. 10% SDS/PAGE analysis of "salt" and "non-salt" fractions.

Salt and non-salt fractions from different levels of purification were compared on SDS/PAGE to demonstrate molecular weight differences of the proteins.

Lane 1. Molecular weight markers

Lane 2. 3-MC, DEAE (salt)

Lane 3. 3-MC, affinity purified

Lane 4. 3-MC, affinity flow through

Lane 5. Blank

Lane 6. BHA, affinity purified

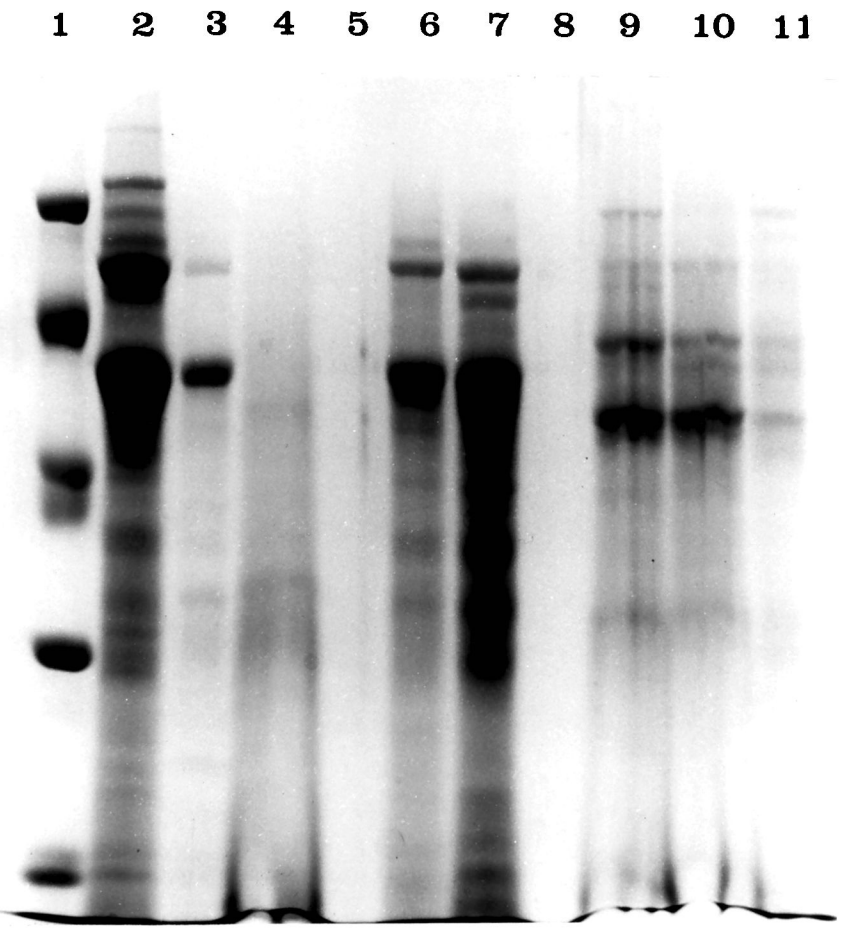
Lane 7. BHA, DEAE (salt)

Lane 8. Blank

Lane 9. BHA, affinity purified (non-salt)

Lane 10. 3-MC, affinity purified (non-salt)

Lane 11. BHA, affinity purified (non-salt)


1
2
3
4
5
6
7
8
9
10
11

To estimate the approximate molecular weight of these protein species, affinity purified samples from BHA, 3-MC and BHA + 3-MC were subjected to SDS/PAGE analysis (Fig. 13). Table 3 shows the relative mobility (R_f) of the protein bands (molecular weight markers and purified UDPGT). From this study, the molecular weights of the salt fraction major and minor bands were estimated to be 57k and 80k, respectively (Fig. 14). The value obtained here for the salt fraction major band is within the accepted range for rat liver p-nitrophenol UDPGT. At this point, the origin of the 80k protein and its relationship to the 57k protein are still unknown.

It has been postulated that UDPGT, in vivo, is a tetramer that may or may not be heterogenous (P. Mackenzie, personal communication). In order to establish the nature of the holoenzyme, and possibly the origin of the 80K protein, affinity purified samples from all the treatments were electrophoresed on a 7.5% native polyacrylamide gel. On this gel only one band was observed in each case (Fig. 15). Interestingly enough, the 80k protein was not detected. This may suggest that the 80k protein is a part of the holoenzyme, and may have the same origin as the 57k protein, therefore co-migrating with it.

Fig. 13. Analysis and determination of subunit molecular weight of affinity - purified UDPGT. Following affinity purification of UDPGT from each treatment group, samples were dialyzed against salt-free elution buffer, concentrated and analyzed by SDS/PAGE at 20 ug of protein per lane.

Lane 1. Molecular weight markers

Lane 2. MC-induced UDPGT

Lane 3. BHA + 3-MC induced UDPGT

Lane 4. BHA induced UDPGT

Lane 5. 3-MC induced UDPGT, affinity flow through

Lane 6. 3-MC induced, pooled peak from DEAE-cellulose

Lane 7. Molecular weight markers

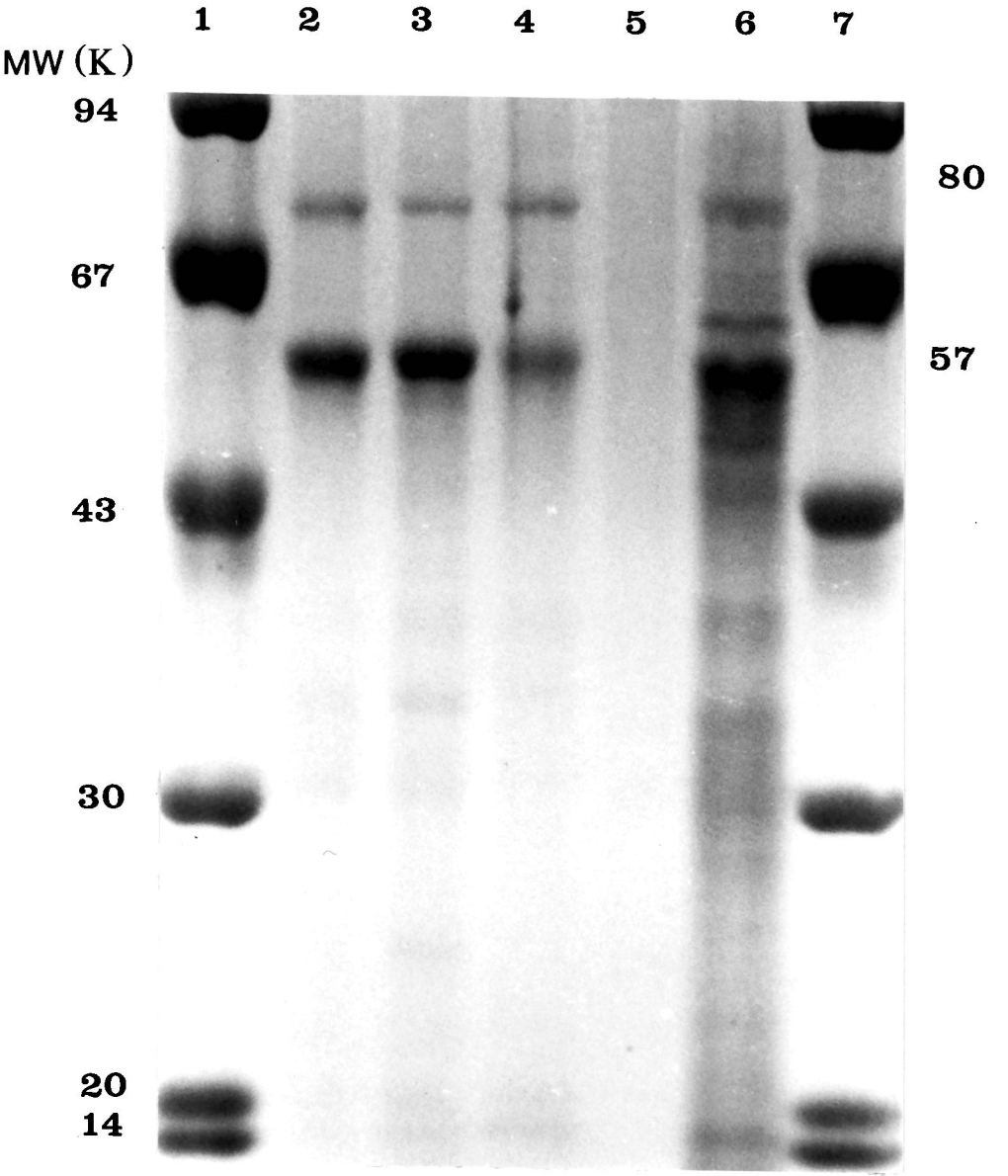

1
2
3
4
5
6
7
MW (K)
94
67
43
30
20
14
80
57

Table 3. P-nitrophenol UDPGT Molecular Weight Determination

Std. Mol. Wt. (k)	Migration Dist. (cm)	Relative Mobility (R_f)
94	3.3	0.24
67	5.0	0.36
43	7.2	0.51
30	7.7	0.55
20	10.0	0.71
14.2	13.3	0.95

Sample	Migration Dist. (cm)	Relative Mobility (R_f)	Molecular Weight (k)
Band 1-UDPGT	4.2	0.30	80
Band 2-UDPGT	6.0	0.43	57

Fig. 14. Estimation of rat liver p-nitrophenol UDPGT molecular weight.

Know molecular weight standards (phosphorylase-b, 94k; Bovine Serum Albumin, 67k; Ovalbumin, 43k; Soybean Trypsin inhibitor, 20k and alpha-lactalbumin, 14k) were separated on a 10% SDS/PAGE, relative mobility determined and plotted on a semi-log paper against the known molecular weights. The molecular weight of UDPGT was estimated from the standard curve base on its relative mobility.

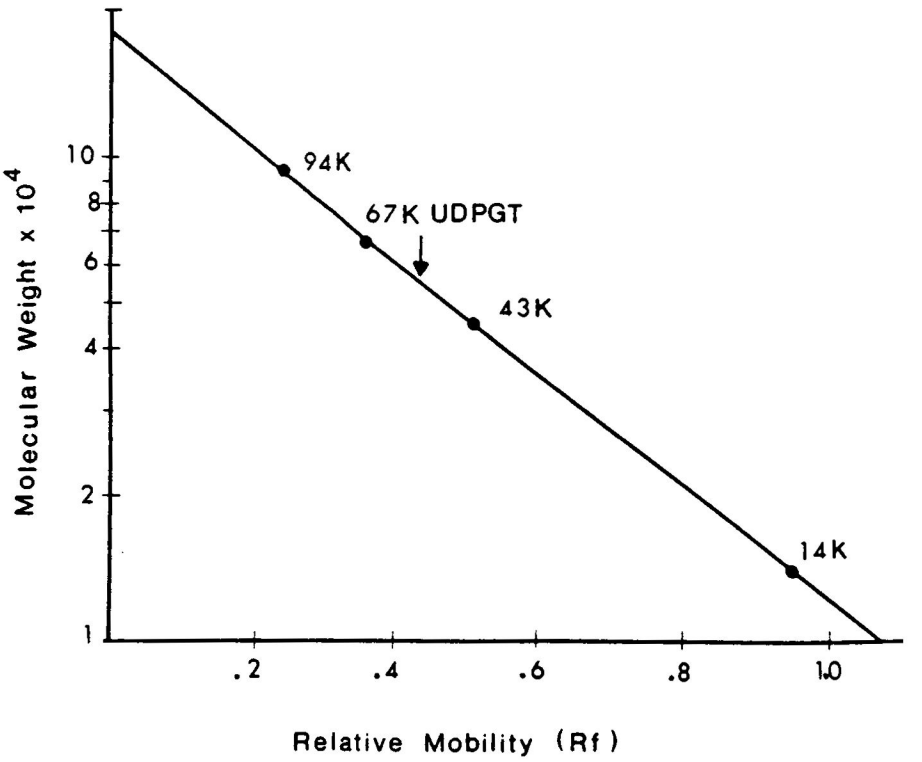

Molecular Weight x 10^4
10
8
6
4
2
1
94K
67K UDPGT
43K
14K
.2
.4
.6
.8
1.0
Relative Mobility (Rf)

Fig. 15. Native gel analysis of affinity purified UDPGT. Affinity purified samples (10 ug) from different treatments were subjected to 7.5% SDS/PAGE analysis to demonstrate the homogeneity of the purified samples, as follows:

Lane 1. Bio-Rad High molecular weight markers

Lane 2. BHA + 3-MC, affinity purified

Lane 3. BHA, affinity purified

Lane 4. 3-MC, affinity flow through

Lane 5. 3-MC, affinity purified

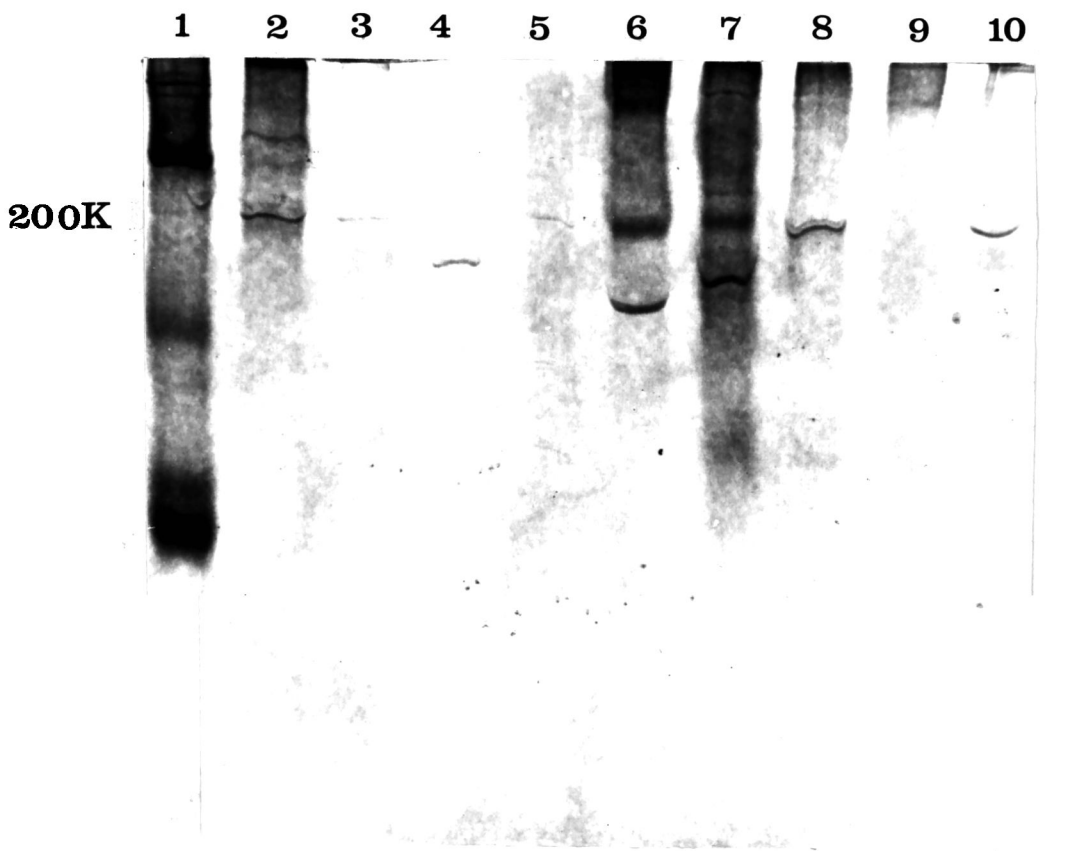
Lane 6. BHA + 3-MC, affinity purified "non-salt"

Lane 7. BHA, affinity purified, "non-salt"

Lane 8. 3-MC, affinity purified, "non-salt"

Lane 9. 3-MC, affinity flow through "non-salt"

Lane 10. Untreated, affinity purified "salt"


1
2
3
4
5
6
7
8
9
10
200K

Elution of the 57K Protein from SDS/PAGE for Antibody Production and Immunological Characterization

Because of the presence of the 80k protein in the partially purified sample, the affinity eluates could not be used directly as a source of antigen for p-nitrophenol UDPGT antibody production. The 80k protein may have been removed by gel filtration, but this was not done since it may have meant losing more of the 57k protein in an extra purification step. To circumvent possible contamination from the 80k protein, the salt affinity eluate from MC treatments was subjected to preparative SDS/PAGE fractionation. The 57k protein was sliced out and mechanically eluted from the gel. Protein concentration was quantitated by the Bradford Method (1976). Two hundred microgram of 3-MC protein in Freund's complete adjuvant was used to immunize New Zealand white rabbits, followed by 150 ug of protein in Freund's incomplete adjuvant booster injections every other week. On the second week, trial serum was collected to test for the presence of UDPGT antibodies against the purified protein by Ouchterlony double immunodiffusion (Fig. 16). Antibody against the 57k eluted from a 3-MC treated animal was detected. When this antibody was reacted against salt samples from all treatments including control and an eluted 80k protein, it cross-reacted with

Fig. 16. Ouchterlony double immunodiffusion: Detection of anti-UDPGT and working dilution determination. The 3-MC 57k protein was used as antigen. The same dilutions were made of the pre-immune serum (B).

Center Well. 57k proten

Well 1. 1:100 dilution

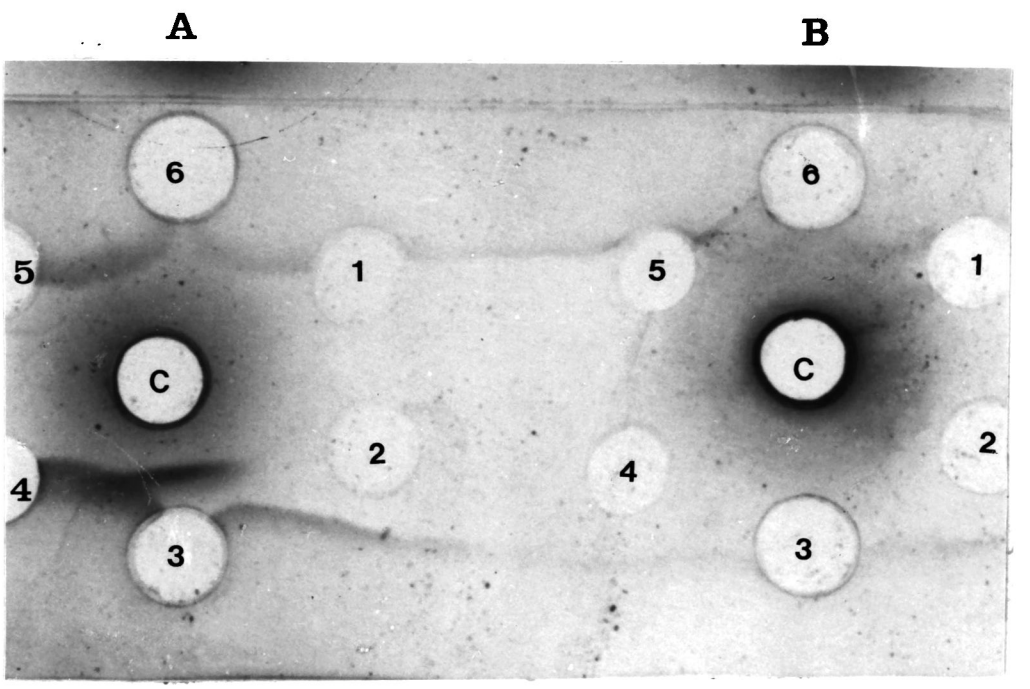
Well 2. 1:20 dilution

Well 3. 1:10 dilution

Well 4. 1:50 dilution

Well 5. 1:200

Well 6. 1:1000


A
B
6
5
1
C
2
4
3
6
5
1
C
2
4
3

all of them. The reactivity with the 80k protein exhibited a spur indicating common and variable sequences between the 57k and 80k proteins (Fig. 17). This observation confirms the initial suspicion that both proteins have a common origin. The cross-reactivity with the BHA and BHA + 3-MC proteins indicates the presence of conserved sequences and common epitopes on the p-nitrophenol forms of UDPGT from these treatments. Although no detectable precipitation line was evident with the UDPGT from the untreated rats, this does not mean the complete absence of these proteins, since the double immunodiffusion assay is not very sensitive.

Having established the presence of p-nitrophenol antibody in the serum, the animals were anaesthesized with a mixture of acepromazine (1.1 mg/kg body weight) and ketamine (22 mg/kg body weight) and bled out by cardiac puncture. The serum was ammonium sulfate precipitated and DEAE-cellulose fractionated (Fig. 18). Three peaks were obtained on the ion-exchange fractionation. The anti-p-nitrophenol UDPGT peak was determined by double immunodiffusion. Most of the anti-p-nitrophenol UDPGT reactivity was located in peak B (Fig. 19).

Antibody titer was determined by double immunodiffusion and later repeated using immunoblot (dot and Western blottings). In the Ouchterlony assays, no precipitation

Fig. 17. Ouchterlony double immunodiffusion analysis of affinity - purified UDPGT from treated and untreated rats against anti-MC UDPGT. UDPGT isolated from different treatment groups and the untreated was tested against rabbit anti-MC UDPGT serum. The center well contained anti UDPGT.

Well 1. PBS

Well 2. UDPGT from untreated rats

Well 3. UDPGT from MC-treated rats (the 57k protein extracted from a 10% SDS/PAGE

Well 4. The 80k protein extracted from a 10% gel

Well 5. UDPGT from BHA + 3-MC treated rats

Well 6. The "non-salt" peak obtained from the DEAE-cellulose chromatography of the MC solubilized microsome.

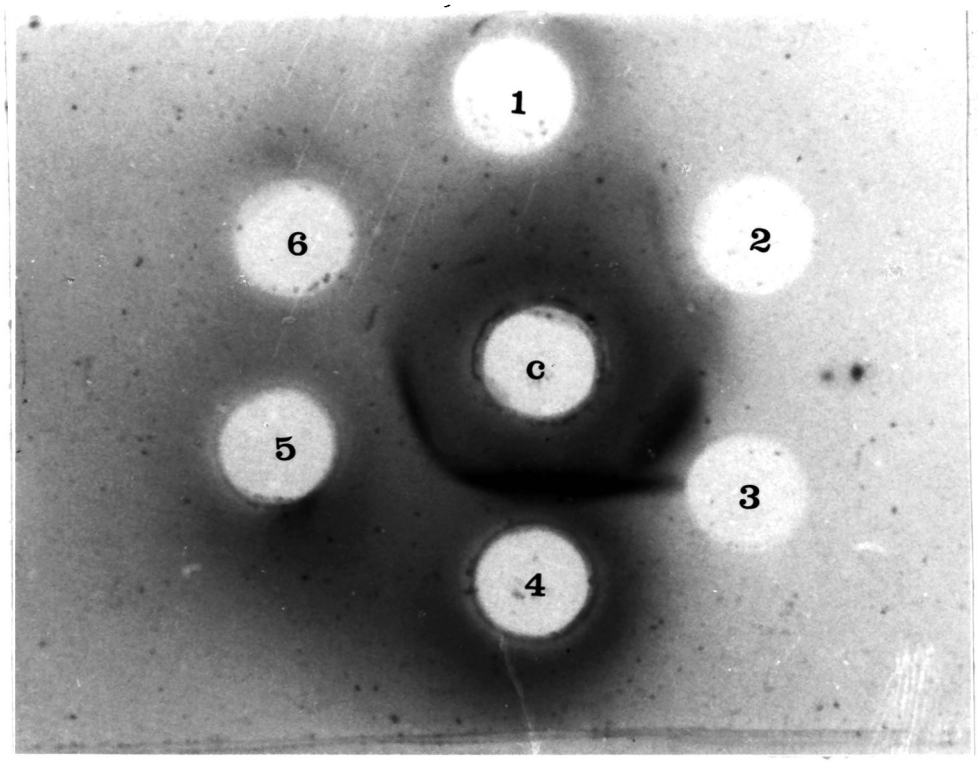

1
6
2
c
5
3
4

Fig. 18. DEAE-cellulose purification of rabbit anti-rat UDPGT serum.
Serum from rabbit that was immunized with the 57k protein from 3-MC induced rat was separated on DEAE-cellulose, and eluted with 0.0175 M phosphate buffer, pH 6.3.

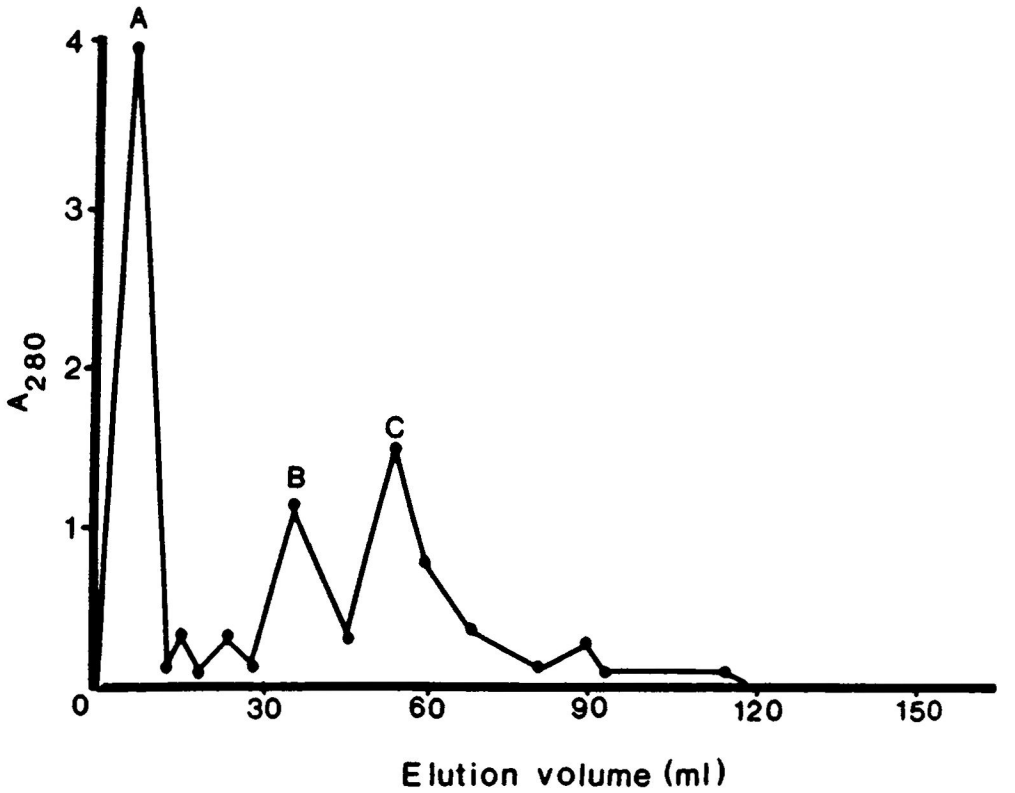

A
B
C
4
3
2
1
0
A_{280}
30
60
90
120
150
Elution volume (ml)

Fig. 19. Ouchterlony double immunodiffusion analysis of DEAE-cellulose purified anti UDPGT. The three proteins peaks obtained from DEAE-cellulose purification were analyzed by double immunodiffusion to ascertain the peak that has anti-UDPGT activity. Center well had the 3-MC 57k protein

Well 1. PBS control

Well 2. Peak A

Well 3. Unpurified serum

Well 4. Peak B

Well 5. Blank

Well 6. Blank

line was observed beyond a 1:10 dilution of the whole serum while a 1:100 and 1:200 dilutions were the limit of the dot and western blots respectively, based on the positive signals observed after a goat anti-rabbit - horse radish peroxidase reaction. Figure 20 shows a western blot analysis of the anti-UDPGT against total rat liver microsomes. A densitometric scan of the dot blot did not show any interpretable data beyond the 1:20 dilution of the antibody (Fig. 21). Based on the titration information obtained, a 1:5 dilution was used as the working dilution in subsequent double immunodiffusion assays, and 1:200 was used in immunoblot assays.

To evaluate the topology of UDPGT on the endoplasmic reticulum membrane, dilutions of the antibody were reacted with solubilized and unsolubilized microsomes, affinity purified proteins from all the treatments including untreated via Ouchterlony immunodiffusion and dot blot assays (Table 4). The solubilized microsomes and the affinity samples reacted as expected. However, the reactivity obtained with the unsolubilized microsomes indicated that UDPGT is a transmembrane protein. Figure 22 shows an immunodiffusion analysis of the unsolubilized microsomes. It seems that the portions outside the membrane contain epitopes that are conserved in the different forms of this

Fig. 20. Western blot analysis of solubilized microsome from 3-MC treated rats.
Different dilutions of solubilized microsome (1.0 mg; 0.75 mg; 0.50 mg and 0.25 mg) were separated on a 10% SDS/PAGE. The proteins were electrotransferred to nitro-cellulose and processed with different dilutions (1:20; 1:200 and 1:1000) of rabbit anti-rat UDPGT serum.

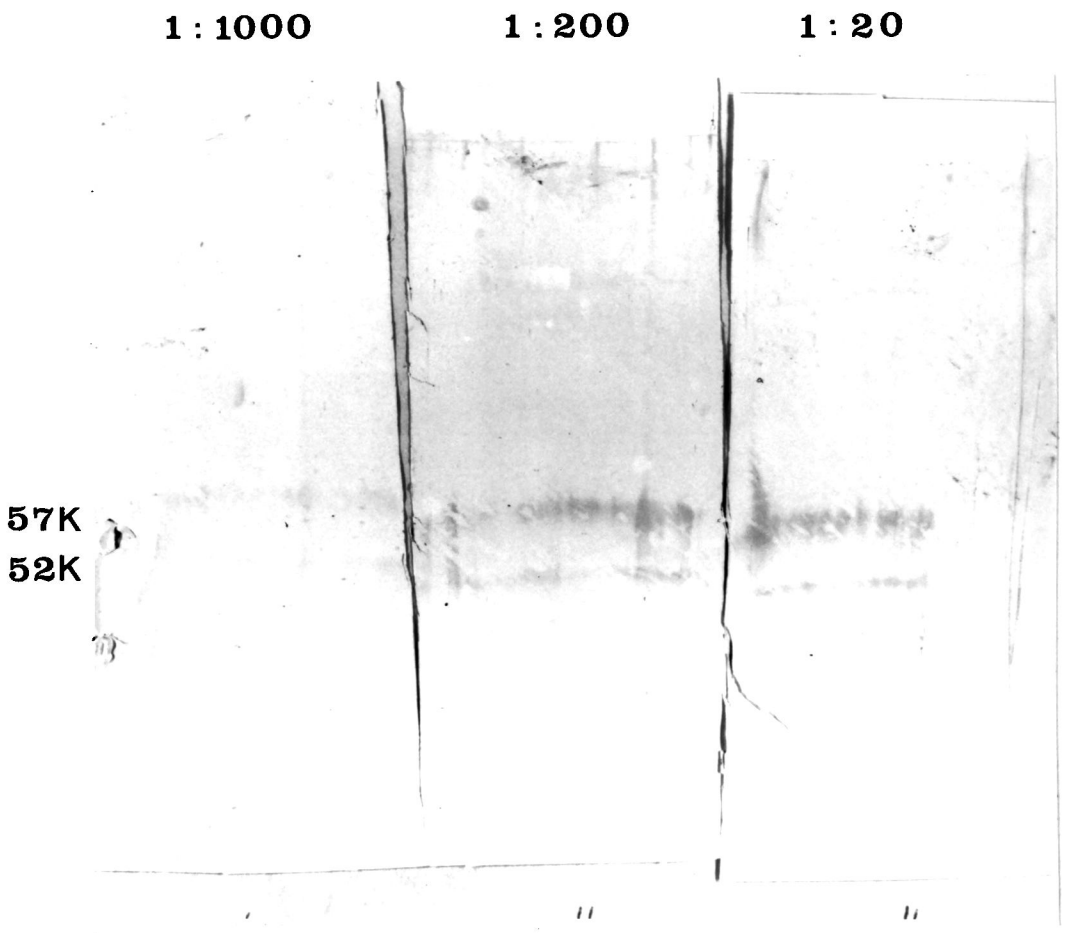
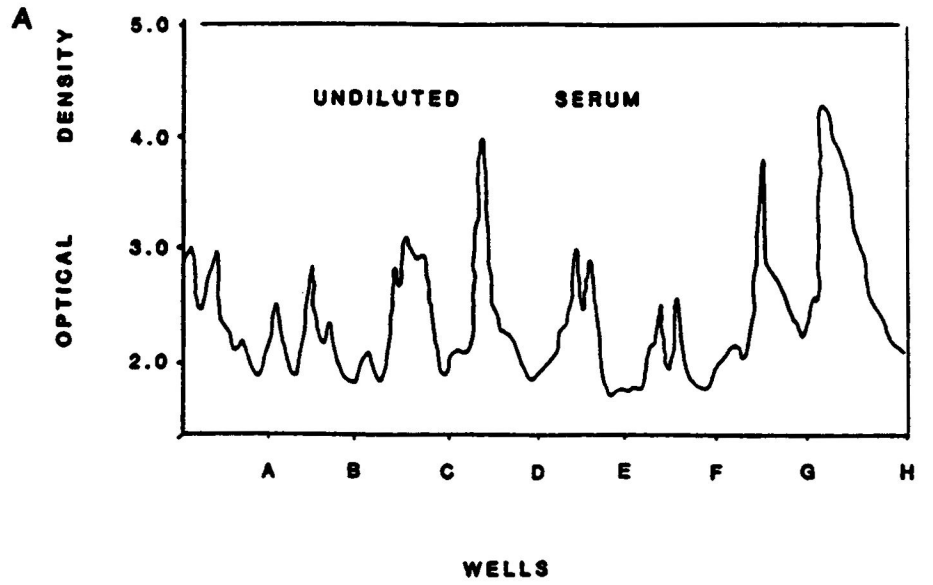

1 : 1000
1 : 200
1 : 20
57K
52K

Fig. 21. Dot-blot analysis and densitometric scan from different treatment groups.

UDPGT from different treatments was added to wells in a 96-well microtiter plate as follows: Columns A and B had untreated UDPGT; C and D had 3-MC UDPGT; E and F had BHA UDPGT, G and H had BHA + 3-MC UDPGT. The samples were dot transferred to nitrocellulose by vacuum suctioning. The nitrocellulose filters were treated with rabbit anti rat UDPGT serum followed by goat anti-rabbit IgG-HRP conjugate. Different dilutions of the antiserum were utilized as follows:

Row 1. Undiluted serum (A)
Row 2. 1:10 serum dilution (B)
Row 3. 1:20 serum dilution (C)
Row 4. 1:100 serum dilution (D)
Row 5. 1:1000 serum dilution (E)


A
OPTICAL DENSITY
5.0
4.0
3.0
2.0
UNDILUTED SERUM
A B C D E F G H
WELLS

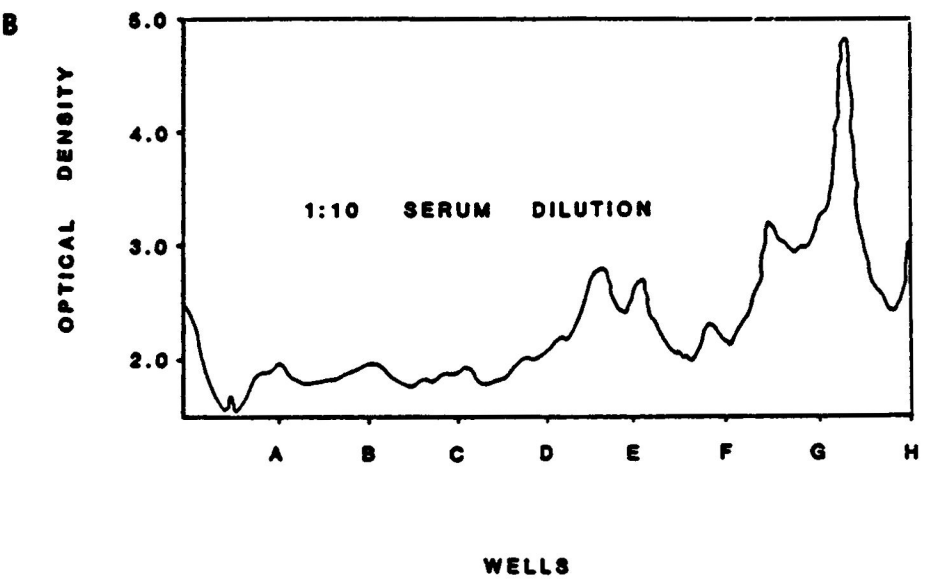

B
OPTICAL DENSITY
5.0
4.0
3.0
2.0
1:10 SERUM DILUTION
A B C D E F G H
WELLS

Fig. 22. Ouchterlony double immunodiffusion of unsolubilized microsome against anti-UDPGT serum. Microsomes from different treatments (50 ug) were immune reacted against anti-UDPGT serum. Center well had rabbit anti-rat 57k protein.

Well 1. PBS control

Well 2. 3-MC microsome

Well 3. Blank

Well 4. BHA microsome

Well 5. Blank

Well 6. BHA + 3-MC microsome

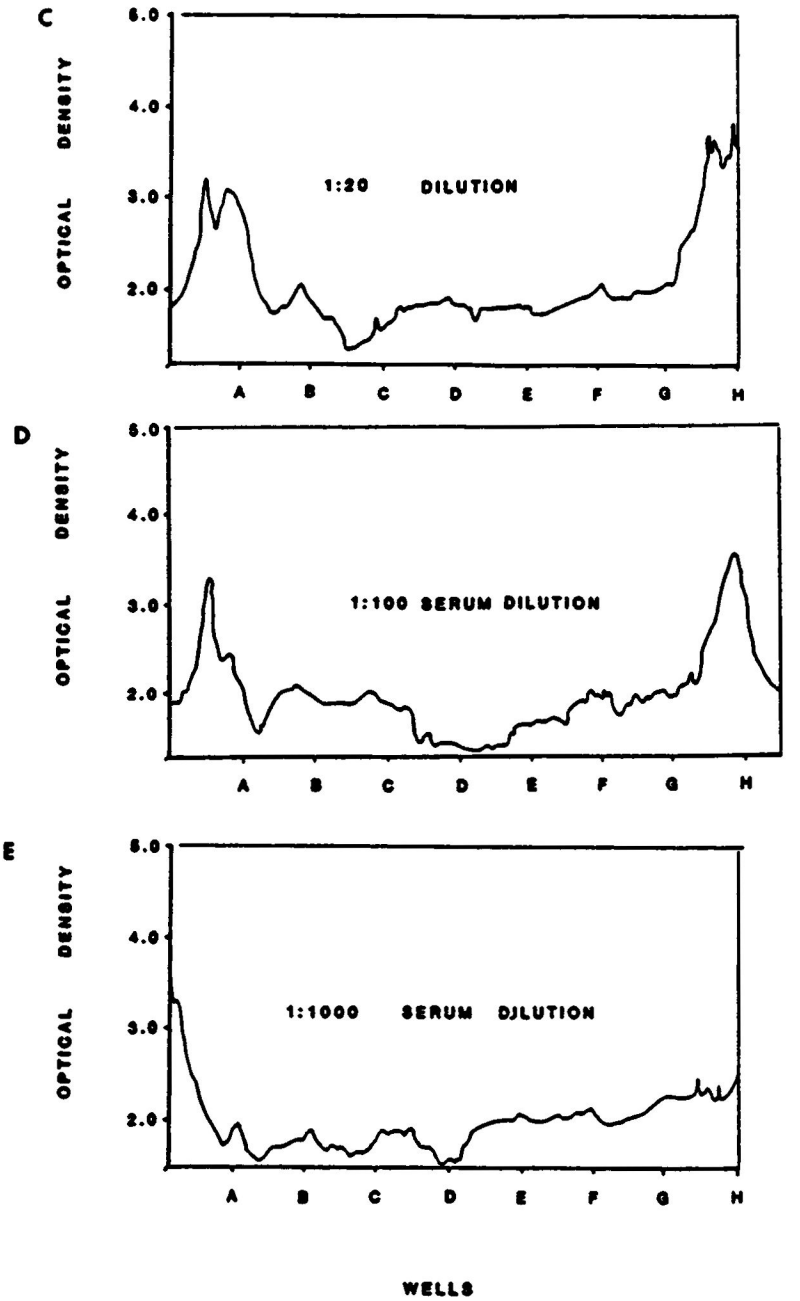

C
OPTICAL DENSITY
6.0
4.0
3.0
2.0
1:20 DILUTION
A B C D E F G H
D
OPTICAL DENSITY
6.0
4.0
3.0
2.0
1:100 SERUM DILUTION
A B C D E F G H
E
OPTICAL DENSITY
6.0
4.0
3.0
2.0
1:1000 SERUM DILUTION
A B C D E F G H
WELLS

Table 4. Reactivity of P-nitrophenol UDPGT from Different Treatments Against Anti-3-MC UDPGT

Antigen Source		Ouchterlony (1:5 dilution)	Immunoblot (1:20 dilution)
Solublized Control	Microsome	+	++
Solublized 3-MC	Microsome	++	++
Solublized BHA	Microsome	++	++
Solublized B+M	Microsome	+++	+++
Unsolublized 3-MC	Microsome	+	++
Unsolublized B+M	Microsome	+	++
Affinity Purified	PNp-UDPGT (Control)	++	++
Affinity Purified	PNp-UDPGT (3-MC)	+++	+++
Affinity Purified	PNp-UDPGT (BHA)	++	++
Affinity Purified	PNp-UDPGT (B+M)	+++	+++
80K Extract from Purified 3-MC		+	ND

\+ = Fairly Intense Reaction

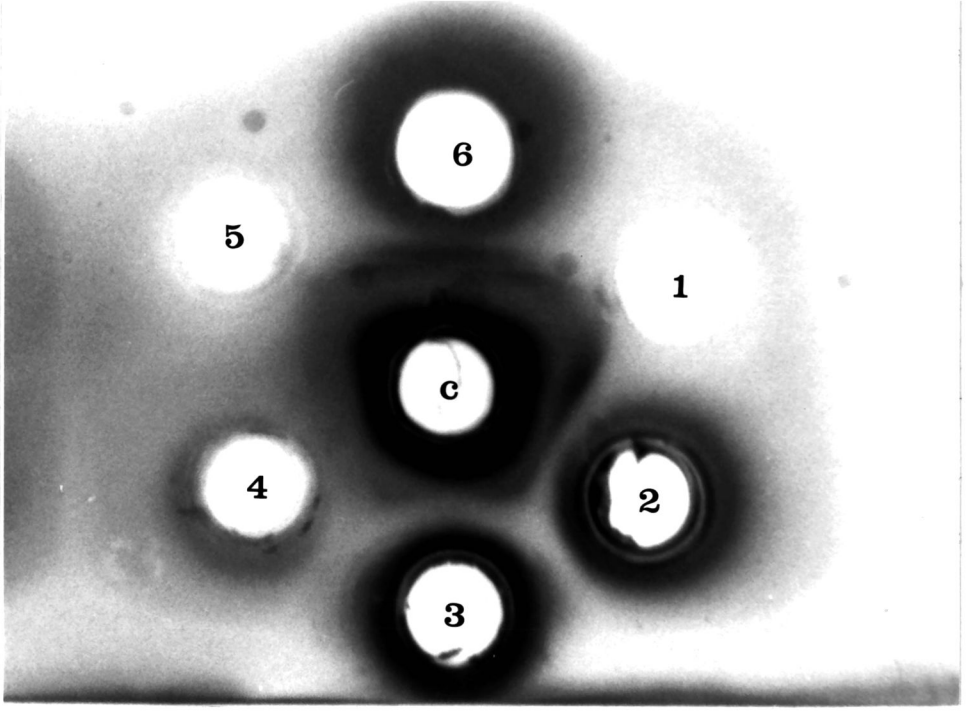
++ = Intense Reaction

+++ = Very Intense Reaction

\- = No Reaction

ND = Not Determined

Fig. 23. Immunoprecipitation of UDPGT activity from reaction mixtures with anti-MC-UDPGT. Series of enzyme reactions were performed in which affinity-purified UDPGT from each treatment group served as the source of enzyme and p-nitrophenol was the substrate used. The percent of anti-MC UDPGT was steadily increased as denoted and the amount of activity remaining was determined for each reaction.


6
5
1
c
4
2
3

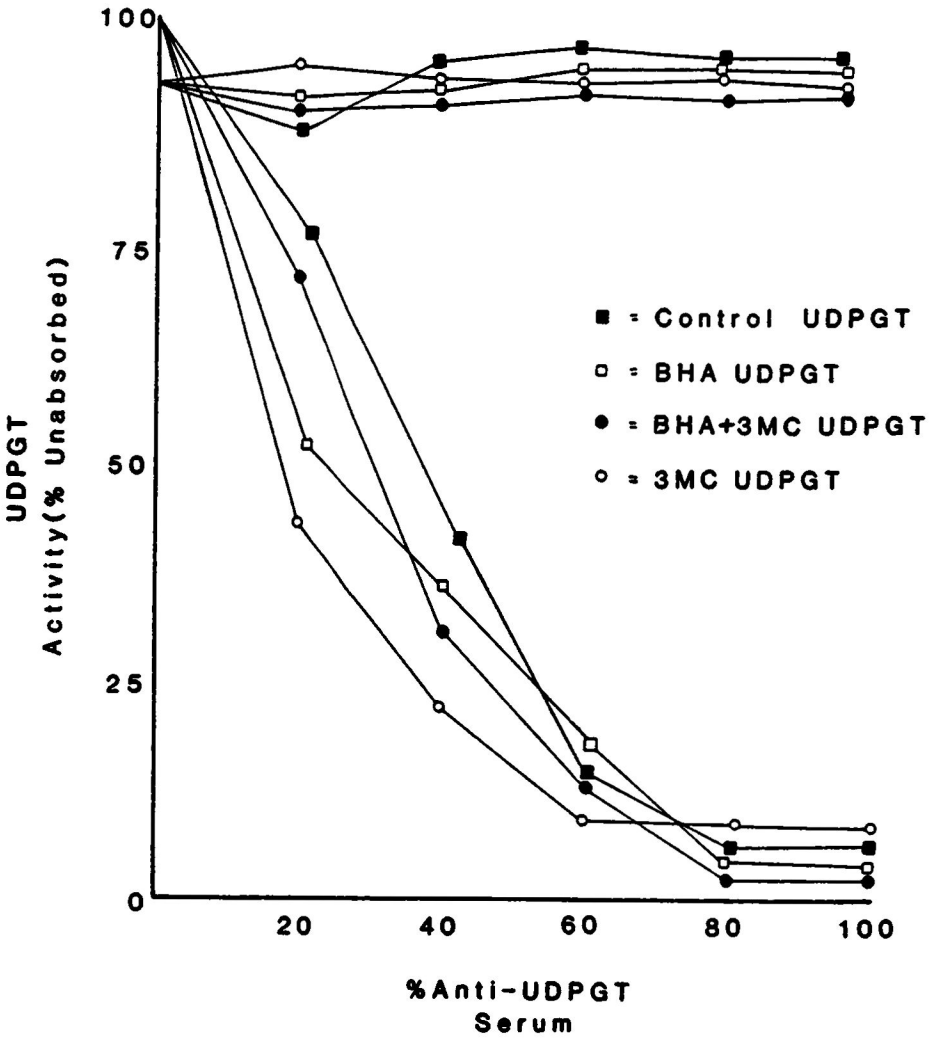
enzyme, and as such could be recognized by antibody made for all forms of the enzyme. The reactivity obtained with the untreated samples was not as intense as those obtained with treated animals. Once again, this is more of an indication of the degree of induction and sensitivity of the assay instead of the nonexistence of this enzyme in untreated animals.

Inhibition of P-nitrophenol UDPGT Activity with Rabbit Anti-57k Serum

Since we know that UDPGT generally exists in multiple forms, and the immunological data obtained here seem to indicate the presence of conserved antigenic determinants on the enzymes from different treatments, it was necessary to deduce the effect of the antibody on the p-nitrophenol UDPGT activity. Secondly, we had to establish that the 57k protein isolated is the enzyme that catalyzes the conjugation of p-nitrophenol to UDP-glucuronic acid. Another important aspect of this study was to demonstrate the relationships among the enzyme from different treatments, considering that the antibody was made against the 3-MC induced 57k protein. To illustrate the specificity of the antibody for p-nitrophenol UDPGT, the experiment was repeated using a rabbit pre-immune serum.

The results (Fig. 23) show that the pre-immune serum could not inhibit the activity in the reaction mixture. About 85% of p-nitrophenol UDPGT activity was retained, irrespective of the treatment and possible enzyme form. However, significant reduction was observed in all the treatments using the specific antisera. The antibody was able to eventually absorb out and inhibit close to 95% of the activity in the reaction mixtures.

To quantitate the inhibition obtained by the immunoprecipitation, the amount of antiserum required to cause 50% inhibition was used as a reference point. It was noted that 20% suspension of the antiserum was required to cause 50% inhibition of the MC-induced UDPGT activity, however, for the same amount of inhibition of the control, BHA and BHA + 3-MC-induced enzymes, the amount of antiserum was 41%, 28% and 32%, respectively. The differential inhibition observed here may reflect the existence of variable amino acid sequences on the different forms of the enzyme. The difference observed between the inhibition of the treated and untreated samples may be indicative of existence of inducer specific forms of UDPGT. This observation may explain the absence of one of the three salt peaks in the DEAE-cellulose fractionation of the untreated samples. Overall, the results show that the 57k is the active p-nitrophenol UDPGT.


100
75
50
25
0
20
40
60
80
100
UDPGT
Activity(% Unabsorbed)
%Anti-UDPGT
Serum
■ = Control UDPGT
□ = BHA UDPGT
● = BHA+3MC UDPGT
○ = 3MC UDPGT

Immunological Comparison of Rat and Mouse UDPGT

Now that we had established that p-nitrophenol UDPGT from different treatments are immunologically identical with subtle variable sequences, it was essential to know if this was a species specific reaction. Goat-antibody against mouse UDPGT (from Dr. Owens, N.I.H) was reacted against the partially purified proteins from the treated animals in an immunodiffusion assay and there was cross-reactivity (Fig. 24). Even though similarities have been demonstrated among the enzymes from different treatments, UDPGT from different species also have conserved sequences.

Tryptic Digest Analysis of UDPGT from Different Treatments

Based on the enzyme immunoabsorption assays in which differential inhibition was noticed, and to verify the possibility of variable sequences on the p-nitrophenol UDPGT from different treatments, the purified enzymes were trypsin digested and the digestion pattern evaluated using HPLC. We were unable to detect the proteolytic fragments by SDS/PAGE. On HPLC, the digestion pattern for the 3-MC and BHA proteins showed regions of identity and some variable regions (Fig. 25). These data gave credibility to the immunological observations which showed cross-reactivity among the different forms, but differential response to the antibody.

Fig. 24. Double immunodiffusion analysis of goat anti-mouse UDPGT and rabbit anti-rat UDPGT against affinity purified rat liver p-nitrophenol UDPGT.

UDPGT from different treatments was reacted against anti-mouse UDPGT serum to demonstrate the interrelationship between rat and mouse UDPGT. Center well (A) = Rabbit anti-rat UDPGT (whole serum). (B) = Goat anti-mouse UDPGT (Ammonium Sulfate ppt).

Well 1. A and B. 3-MC, affinity purified UDPGT

Well 3. A and B. BHA, affinity purified UDPGT

Well 5. A and B. BHA + 3-MC, affinity purified UDPGT

The rest of the wells were blank.

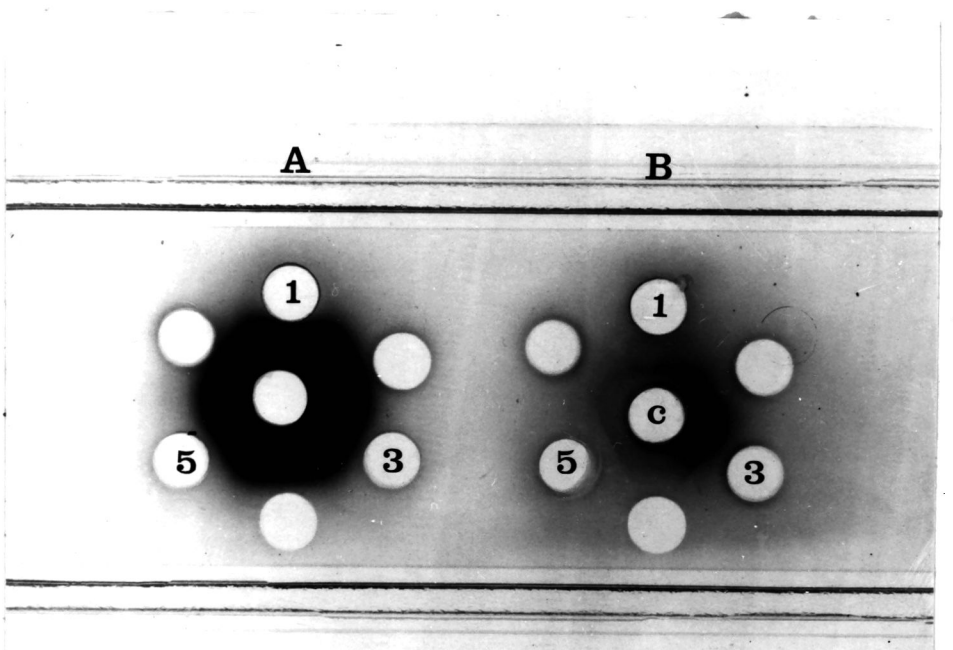
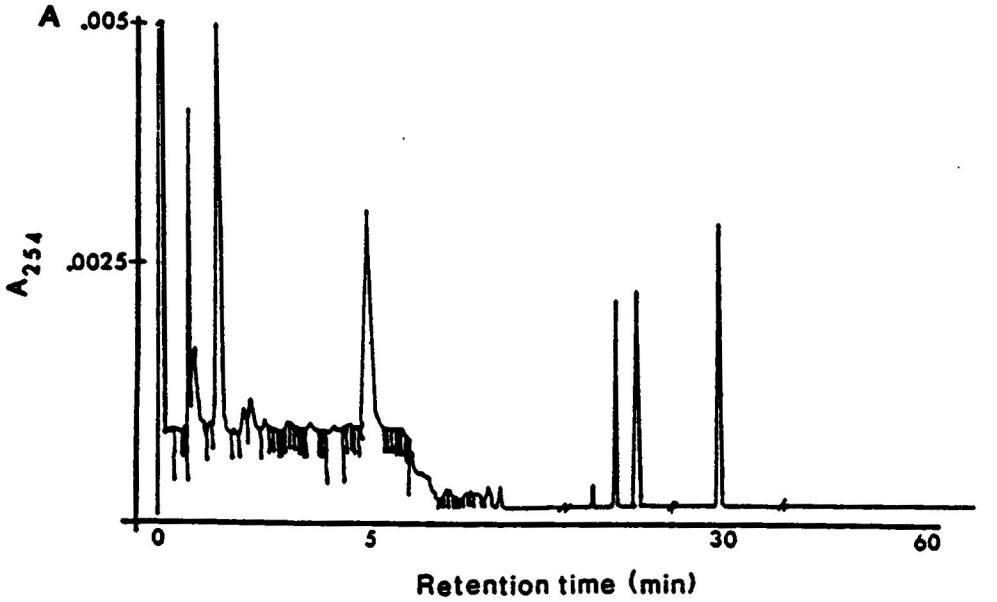

A
B
1
5
3
1
c
5
3

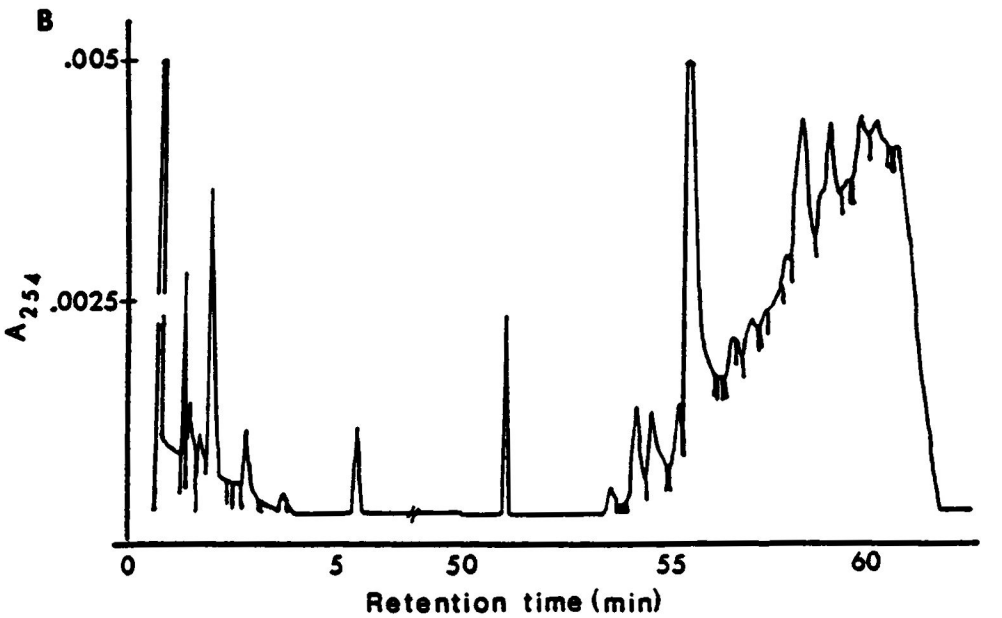
Fig. 25. Tryptic digest analysis of UDPGT from 3-MC and BHA induced rats.

Fifty microgram of UDPGT from BHA and 3-MC induced rats were trypsin digested, and the fragmented separated on HPLC.

A. 3-MC, affinity purified

B. BHA, affinity purified


A
.005
.0025
A_{254}
0
5
30
60
Retention time (min)


B
.005
.0025
A_{254}
0
5
50
55
60
Retention time (min)

mRNA Isolation and Characterization

Consistently, we have observed an elevation of the UDPGT activity in induced animals. It was essential at this point to know if the increase in enzyme activity was due to the activation of pre-existing enzymes or activation of the gene by the inducers. In addition, we needed to confirm that the 57k protein could be obtained from *in vitro* translation and how it compared with the native *in vivo* translated protein. Poly(A^+) RNA was obtained by the guanidine thiocyanate method from a BHA + 3-MC treated rat liver (Table 5). From 10g liver tissue, 50 ug/ml of mRNA was isolated. Here, we noticed an 80% increase in the amount of total RNA in induced compared to 20% in uninduced animals (Fig 26). The initial indication is that the elevated activity may be a transcriptional phenomena instead of a post-translational event.

The *in vitro* translational assay was performed with 2.0 ug of the poly(A^+) RNA in nuclease treated rabbit reticulocyte. Dog pancreatic microsomes were added to the reaction mixture to simulate the *in vivo* processing conditions in which pre-UDPGT is cleaved to mature UDPGT by removing the signal sequences. The reaction mixture was immunoprecipitated using rabbit antibody against 57k p-nitrophenol UDPGT, followed by protein A-sepharose precipitation. Table 6 shows [^{35}S]-Methionine incorporation that was measured using

Table 5: Poly (A^+) RNA Isolation

Sample	A_{260}	A_{280}	260/280	Dilution Factor	[RNA] ug/ml	% Recovery	% Efficiency
Guanidium Thiocyanate Extract	2.280	2.744	0.831	1:100	9120	100	100
Phenol Extract	2.160	2.622	0.824	1:100	8640	95	95
Cesium Chloride Pellet	2.019	2.576	0.784	1:100	8436	93	98
First ETOH Precipitate	0.426	0.262	1.626	1:100	1704	19	20
Second ETOH Precipitate	0.408	0.274	1.489	1:100	1632	18	96
Oligo (dT) Fraction	0.639	0.476	1.342	1:10	256	3	16
Oligo (dT) Phenol Extract	0.126	0.087	1.448	1:10	50	0.5	20

Fig. 26. Isolation of total hepatic RNA from each treatment group.

The quantity of total hepatic RNA was determined for each group using 10g of tissue. RNA was quantitated based on the assumption that A_{260} of 1 = 40 ug/ml RNA

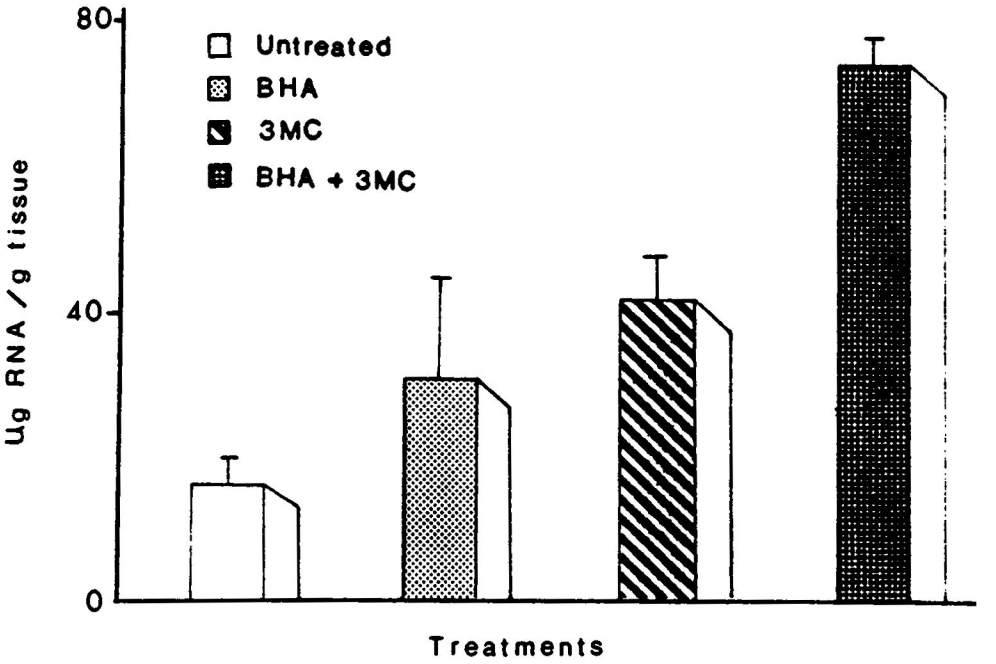

80
40
0
Ug RNA / g tissue
Untreated
BHA
3MC
BHA + 3MC
Treatments

Table 6. *In Vitro* Translation and Immunoprecipitation of Rat Liver P-nitrophenol UDPGT (BHA + 3 MC mRNA).

Sample	Total CPM	CPM after Immunoprecipitation
Total Rat Liver mRNA	5749	1395
Globin mRNA	3760	240*

*Globin mRNA *in vitro* translation was immunoprecipitated with anti-p-nitrophenol UDPGT.

a scintillation counter. Here again, the antibody was able to precipitate out the 80k and 57k as the major proteins in both the in vivo translated samples. (Fig. 27a). Only the 57k of in vitro translated sample that had [^{35}S]-methionine incorporated was detected in the autoradiogram (Fig. 27b). The in vitro translated sample with dog pancreate microsome had the same electrophoretic mobility as the 57k protein of in vivo translated samples (Fig. 27a). The initial indication was that the 57k protein is a bonafide rat liver protein whose mRNA is represented in the population of mRNA we have isolated. With this total rat liver mRNA, we were ready to construct cDNA for library generation.

To further demonstrate that increased activity in induced animals was a transcriptional event, we isolated poly(A^+) RNA from the livers of untreated, BHA, 3-MC and BHA + 3-MC treated rats. The data correspond stoichiometrically with the enzyme activity and protein accumulation results. BHA + 3-MC showed an additive accumulation of poly(A^+) RNA compared to either 3-MC or BHA alone (Fig. 26). This is further evidence that gene activation is involved in the observed over expression of UDPGT mRNA.

Fig. 27a. Comparison of *in vitro* and *in vivo* translated UDPGT.

Poly(A^+) RNA was isolated from a BHA + 3-MC treated rat, and 2.0 ug was used in cell-free protein synthesis in the presence of dog pancreatic microsomes, and [^{35}S]Methionine. *In vitro* translated as well as *in vivo* translated UDPGT were immunoprecipitated, and subjected to SDS/PAGE analysis.

Lane 1. *In vivo* 3-MC UDPGT

Lane 2. *In vivo*, untreated UDPGT

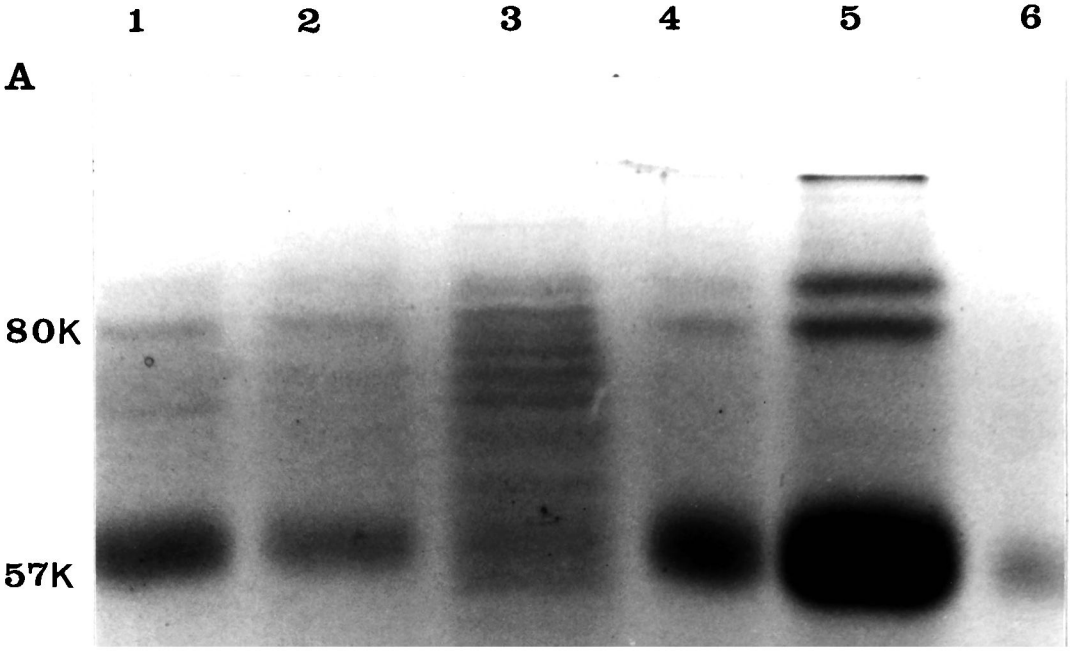
Lane 3. Commercial globin mRNA

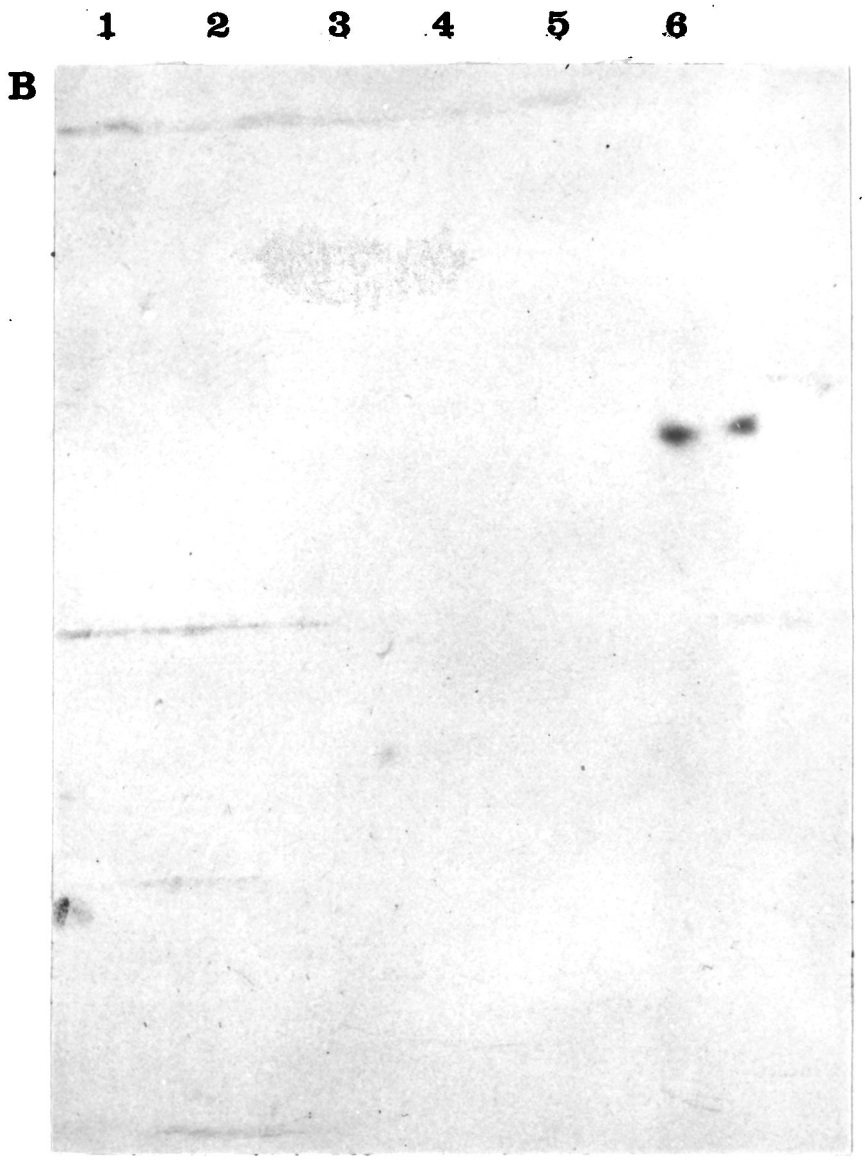
Lane 4. *In vivo*, BHA UDPGT

Lane 5. *In vivo*, BHA + 3-MC UDPGT

Lane 6. *In vitro* translated poly(A^+) RNA

Fig. 27b. Autoradiogram of Fig. 27a.


1
2
3
4
5
6
A
80K
57K


1
2
3
4
5
6
B

cDNA Construction from Total mRNA and Cloning into pBR322

By the reverse transcriptase method, single stranded cDNA was constructed from the isolated total rat liver poly(A^+) RNA that was isolated from BHA + 3-MC treated rats. From 10 ug of poly(A^+) RNA in a 50 ul reaction mixture, 0.910 ug of single stranded cDNA was obtained for an efficiency of 9% (Table 7). Using 0.91 ug of the first strand cDNA, 0.63 ug of the double stranded cDNA was generated for a recovery rate of 69%. This double stranded cDNA was d(C) homopolymer tailed using terminal deoxytransferase and cloned into the PstI site of pBR322 that has been tailed with poly d(G). Following phenol-chloroform extractions and ethanol precipitation, the pBR322 carrying these cDNA inserts were used to transform E. coli HB101 which was plated on TYE agar that contained 15 ug/ml tetracycline. The plates were incubated for 48 h and the colony forming units counted. The number of transformants observed was not above background (Table 8), and the transformation efficiency was relatively low. Based on this result, we decided to repeat the process using the expression vector (Lambda gt11) instead of pBR322 and for the ease of screening the library that may be generated. Furthermore, we chose to use specific mRNA isolated by polysome immunoadsorption instead of total rat liver mRNA hoping that our

Table 7. Total Rat Liver cDNA Synthesis

Sample	Starting Concentration (ug)	CPM	Yield (ug)	Efficiency (%)
Poly(A^+) RNA	10.0	165	-	-
Single Strand cDNA	10.0	2.76×10^6	0.0914	9.14
Double Strand cDNA	0.910	2.39×10^5	0.632	69

Table 8. Total Rat Liver cDNA Library Production: Plasmid pBR-322 Transformation of *E. coli* HB 101

Treatment	Colony Forming Units (CFU/ml)
HB 101 Inoculum	8.0×10^5
Culture at Harvest	1.0×10^8
Competent Cell without DNA	<10
Uncleaved pBR322 (100 ug)	1.0×10^2
Cleaved pBR322 (G-tailed)	<10
pBR322 - Rat Liver cDNA Chimera	0.7×10^1
pBR322 - Globin cDNA Chimera	0.5×10^1

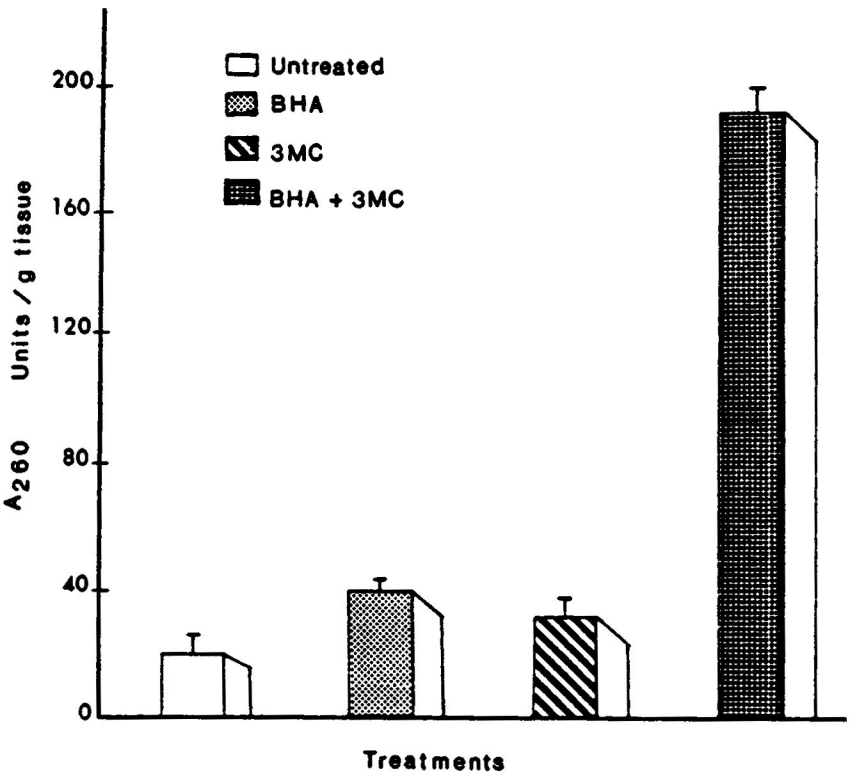
chances of isolating the clones carrying the p-nitrophenol UDPGT gene would be increased.

Isolation of Poly(A^+) RNA by Polysome Immunoadsorption

If the induction that has been observed in this study is a transcriptional event, some difference should be observed in the level of polysome obtained from the different treatments. Using a modification of the Kraus and Rosenberg (1982) technique as was described by El Dorry, (1986); Giachelli and Omiecinski (1987), polysomes were isolated from 5 g of rat liver of all the treatments and the untreated. The level of polysomal RNA accumulation was consistent with the level of protein and specific enzyme activity that was observed earlier. Statistically, there was no significant difference between the level of induction obtained from BHA treatment compared to 3-MC ($p < .01$). However, a synergistic accumulation of polysomal complex was observed with the BHA + 3-MC treatment compared to the single treatments as shown in Fig. 28. The treated samples showed more polysomal complex accumulation than the untreated control. This result further substantiates that these xenobiotics can modulate gene expression.

Fig. 28. Isolation of polysomes from rat liver of each treatment group.

Homogenized rat liver tissue (5 g) from each treatment was loaded onto 1.4 M sucrose and centrifuged. The polysome pellet was resuspended in polysome buffer containing heparin and sodium chloride.


Untreated
BHA
3MC
BHA + 3MC
200
160
120
80
40
0
A260 Units / g tissue
Treatments

From these polysomes, UDPGT specific mRNA was isolated by immunoadsorption, followed by repeated cycles of oligo (dT) chromatography. Specific poly(A^+) RNA from a 3-MC treated rat was used in an *in vitro* translation assay and compared to total poly(A^+) RNA from all the treatments. The translation mixture was subjected to immunoprecipitation before application to a 10% SDS/PAGE analysis.

The result showed two major protein bands for all the samples irrespective of the treatments or the source of the mRNA. The low and high electrophoretic mobility bands were estimated to be about 60k and 52k respectively. A minor protein band was also observed in the 82 - 84k range (Fig. 29). The 60k and 83k protein bands obtained here are consistent with the 57k and the 80k protein obtain from the *in vivo* translated samples considering that these samples were neither glycosylated nor processed to cleave the signal sequence. The 52k protein obtained here was interpreted to be another form of UDPGT as was noticed when the *in vivo* translates were affinity fractionated and SDS/PAGE analysed. When this result was compared to samples that were not immunoprecipitated, these bands were not as pronounced, using coomassie blue stain. The intensity of the bands was increased by silver staining. Even though the intensity of the bands was not as pronounced, more protein bands

Fig. 29. SDS/PAGE analysis of cell-free synthesized and immunoprecipitated UDPGT.

Poly(A^+) RNA was extracted from the liver of a rat in each treatment group and used for *in vitro* translation in the presence of [^{35}S]-methionine. Anti-MC-UDPGT was used for all immunoprecipitation. Lanes were as follows:

Lane 1. Molecular weight markers

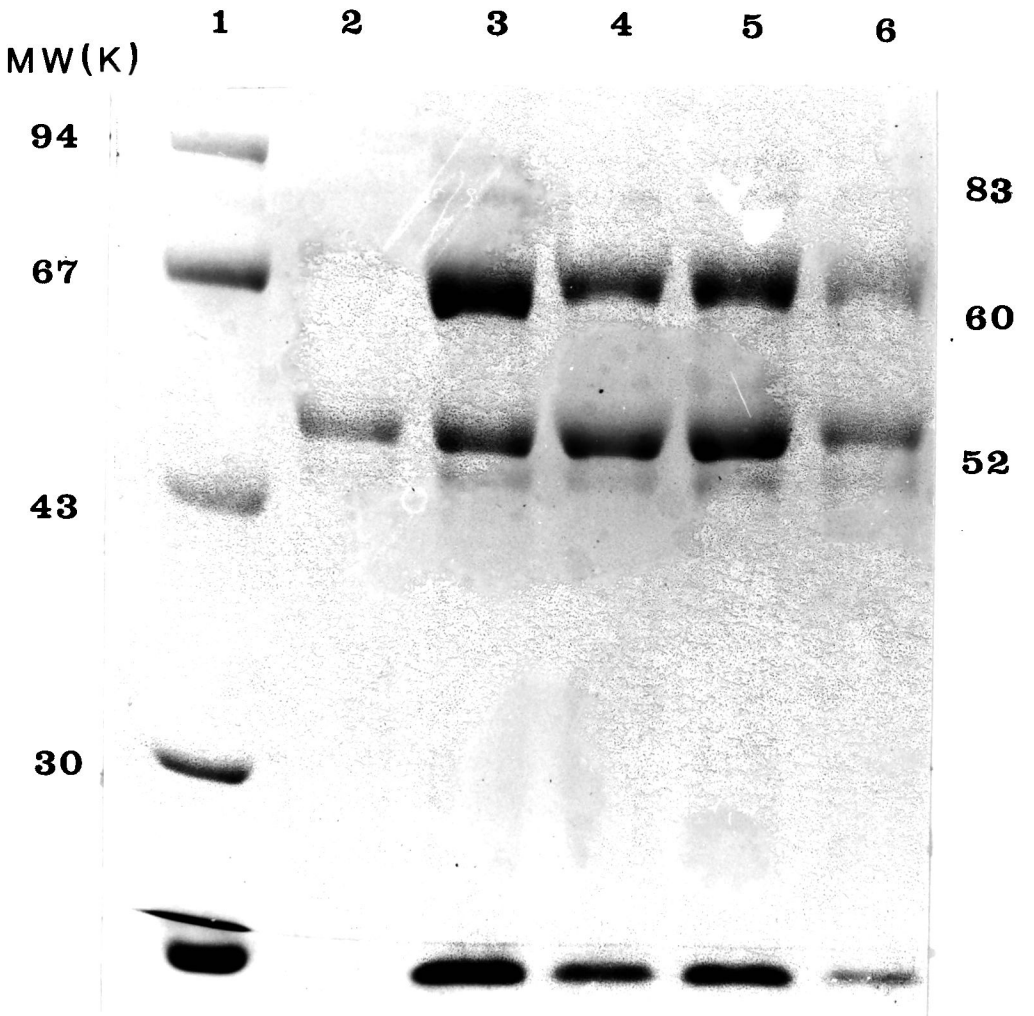
Lane 2. BHA + 3-MC, UDPGT polysomes

Lane 3. BHA + 3-MC, translated from poly(A^+) RNA

Lane 4. BHA UDPGT, from poly(A^+) RNA

Lane 5. 3-MC UDPGT, poly(A^+) RNA

Lane 6. Immunoadsorbed polysome: Poly(A^+) RNA extraction (3-MC UDPGT specific mRNA)


1
2
3
4
5
6
MW(K)
94
67
43
30
83
60
52

were observed compared to the immunoprecipitated. It seems from these data, that the immunoprecipitation utilized above selected against the non-UDPGT proteins, thereby making it possible for us to concentrate the p-nitrophenol UDPGT in these samples. The precipitated and non-precipitated samples that had been electrophoresed on 10% SDS/PAGE were subjected to autoradiography to detect the protein bands with [^{35}S]-methionine labels. More low molecular weight proteins were observed in the unprecipitated samples which were not detected in the precipitated samples (Fig. 30). These proteins are outside the accepted molecular weight range for p-nitrophenol UDPGT and are not recognized by the polyclonal antibody raised against the 57k protein which has been demonstrated to react with other forms of UDPGT because of the conserved nature of the protein family.

To demonstrate that the differential accumulation of the polysomal complex in these treatments correlates with the amount of specific poly(A^+) RNA that could be induced, mRNA was isolated from these polysomes and purified by oligo (dT) chromatography. Fig. 31 shows the differential induction of UDPGT specific mRNA according to treatments. While no considerable difference was observed between the mRNA obtained from BHA or 3-MC treated animals, an additive induction was obtained from the BHA + 3-MC treated animals.

Fig. 30. Autoradiogram of *in vitro* translated, but non-immunopreciptated poly(A^+) RNA from treated rats.
Poly(A^+) RNA was isolated and *in vitro* translated without dog pancreatic microsome. [^{35}S]methionine was used to label the proteins. SDS/PAGE was performed, the gel dried and autoradiographed,

Lane 1. BHA + 3-MC, polysome
Lane 2. BHA + 3-MC, poly(A^+) RNA
Lane 3. 3-MC, poly(A^+) RNA
Lane 4. BHA, poly(A^+) RNA
Lane 5. 3-MC, polysomes
Lane 6. 3-MC, UDPGT specific poly(A^+) RNA
Lane 7. BHA, polysome

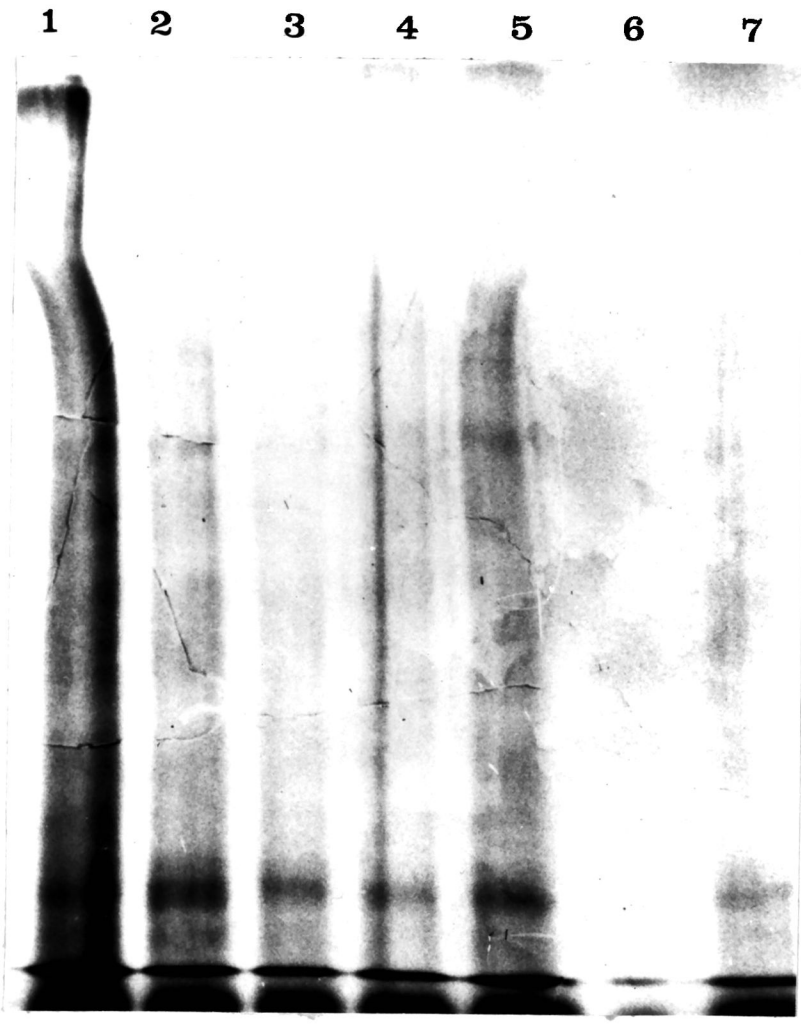
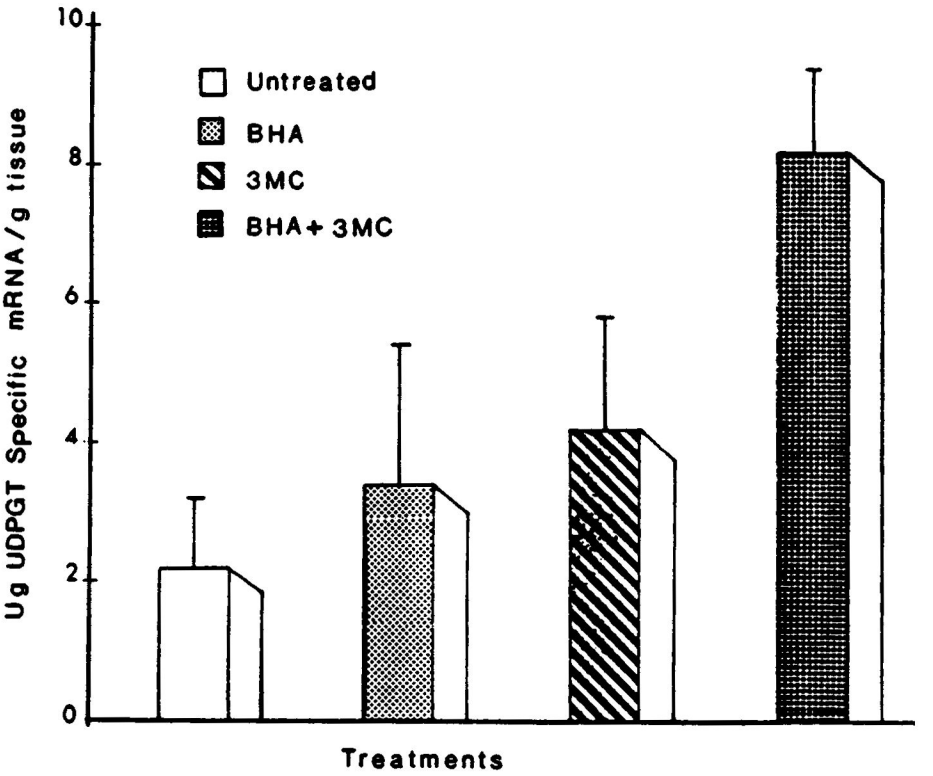

1
2
3
4
5
6
7

Fig. 31. Isolation of UDPGT specific mRNA by polysome immunoadsorption.

Isolated polysomes were reacted with anti-UDPGT and the binary complex loaded onto Pro-A sepharose. The total polysomal RNA was eluted with EDTA, and the specific mRNA isolated by oligo (dT) chromatography.


10
8
6
4
2
0
Ug UDPGT Specific mRNA/g tissue
Untreated
BHA
3MC
BHA+3MC
Treatments

These data further validate the statement that increased enzyme activity observed earlier was not an activation event, but rather a result of gene expression.

cDNA Synthesis from UDPGT Specific mRNA

Double stranded cDNA was constructed starting with 1.0 ug of mRNA from each treatment, using globin mRNA as a positive control. The first strand was labelled with [^{35}S]-ATP and the total count was about 1.50 x 10^7 CPM. The average specific activity was 2.74 x 10^5 cpm/ug RNA, 2.66 x 10^5 cpm/ug RNA, 1.80 x 10^5 cpm/ug RNA and 2.48 x 10^5 cpm/ug RNA for BHA, 3-MC, BHA + 3-MC and globin control, respectively. The first strand synthesis efficiency was 9.0%, 12.1%, 17.4% for BHA, 3-MC and 3-MC and BHA + 3-MC, respectively. These results were comparative to the efficiency obtained with globin mRNA (10.2%). Table 9 shows a comparative illustration of the synthesis of cDNA and the yield from the treatments.

When the first strands were used as template to synthesize the second strand, the percent recovery was 78,69, 62 and 83 for BHA, 3-MC, BHA + 3-MC and globin, respectively. These numbers were within the expected range for second strand cDNA synthesis.

Table 9. cDNA Synthesis from UDPGT Specific mRNA

Sample (1.0 ug/20 ul)	Zero Time Unprecipitated	1st Strand				2nd Strand			
		CPM/2 ul	Total Count	ss cDNA (ug)	Efficiency (%)	CPM/2ul	Total Count	ds cDNA (ug)	Recovery (%)
BHA	1.70×10^7	$.099 \times 10^7$	2.47×10^4	0.090	9.00	1558	1.94×10^4	0.142	78.0
3-MC	1.65×10^7	$.129 \times 10^4$	3.22×10^4	0.121	12.10	1803	2.25×10^4	0.169	69.0
BHA + 3-MC	1.12×10^7	$.126 \times 10^4$	3.14×10^4	0.174	17.40	1553	1.94×10^4	0.216	62.0
Globin	1.54×10^7	$.101 \times 10^4$	2.53×10^4	0.102	10.20	1686	2.11×10^4	0.170	83.0

NOTE: 50 uCi/5 ul of [^{35}S]-ATP (800 ci/mMole) was used in each 60 ul reaction mixture.

Furthermore, these cDNAs were methylated with EcoR1 methylase so that they could be used to clone into the EcoR1 site of Lambda gt11 to generate a rat liver cDNA library.

Screening the Rat Liver cDNA Library for UDPGT Clones.

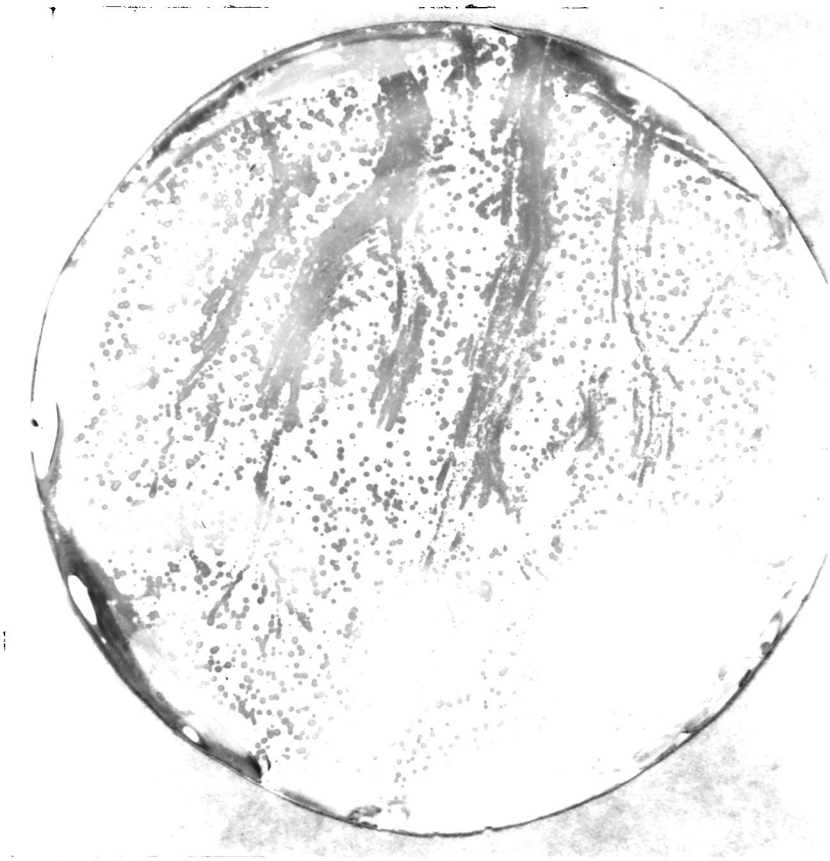
About 200,000 lambda gt11 plaques were screened to isolate the clone(s) carrying UDPGT inserts. We obtained thirty-five positive clones. By a random check, one out of every three (33%) of these turned out to be true positives after they have been plaque purified (Fig. 32). DNA has been isolated from these phages which are to be characterized in future experiments to determine the insert size and their identities in terms of the gene product expressed.

Selection of Monospecific Antibodies Recognizing UDPGT cDNA Carrying Recombinants and Western Blotting

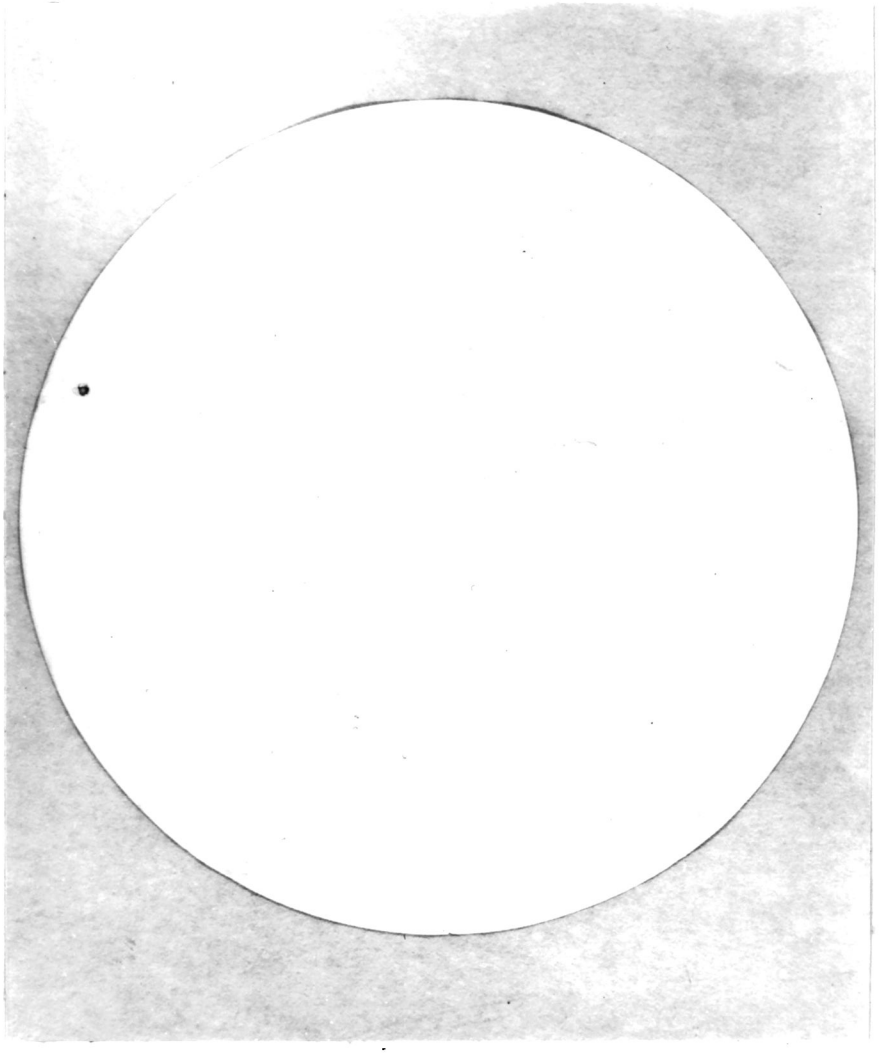
Since the antibody we generated cross-reacts with all UDPGT forms, 3-MC UDPGT specific antibodies were purified from the polyclonal antiserum by the antibody select technique of Barlow et al. (1987). The affinity purified antibodies were used to analyze solubilized rat liver microsomal proteins that had been separated by SDS gel electrophoresis. The antibodies recognized only UDPGT from 3-MC

Fig. 32. Immunoscreening and plaque purification of Lambda gt11 cDNA library.
Lambda gt11 cDNA library was screened to select those clones with UDPGT inserts. The positive plaques were plaque purified to select (A) true positive clones and (B) negative clones.

A



B

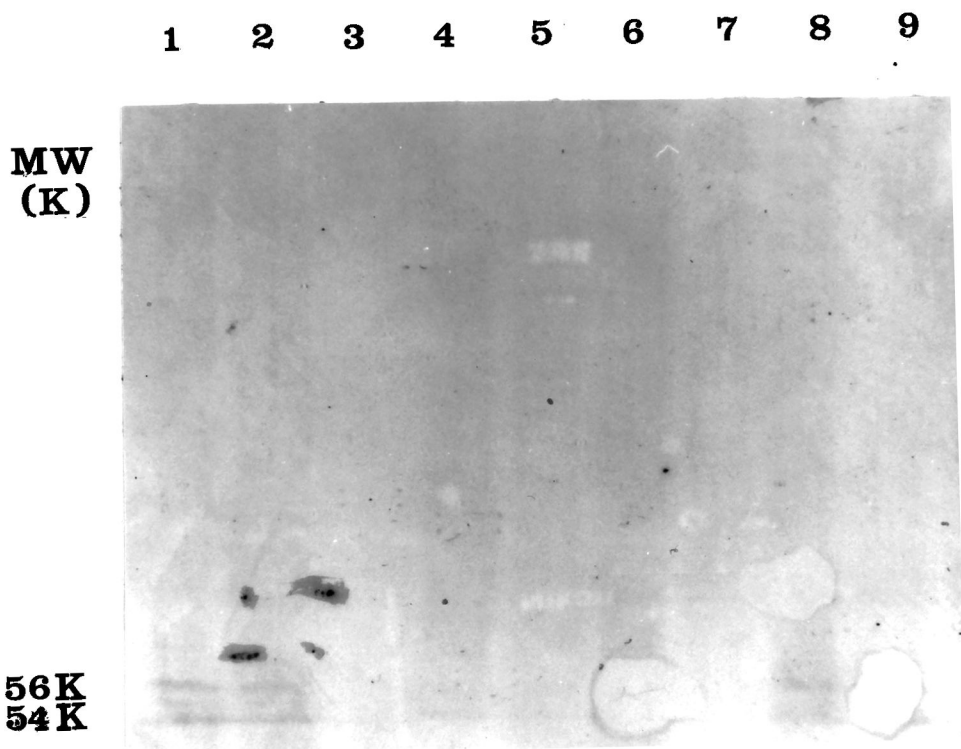


and BHA + 3-MC treated animals. This suggests that the population of antibodies that recognized 3-MC forms of p-nitrophenol UDPGT were selected (Fig. 33) as opposed to those that recognized the BHA forms. The intensity of the positive bands was more pronounced in the combined induction of BHA + 3-MC compared to 3-MC alone, which is consistent with earlier results obtained when enzyme activity, protein accumulation and specific mRNA were evaluated. Moreover, two major bands that are 57K and 52K were obtained from the positive reactions, which correspond to the anionic and cationic forms of UDPGT that were demonstrated in the enzyme purification analysis. The ability of these monospecific antibodies to select against BHA forms of the enzyme is another indication for the presence of unique and variable sequences on different forms of UDPGT since the polyclonal antibody was made against a 3-MC induced protein.

Fig. 33. Antibody selection of inducer specific forms of UDPGT.

Positive clones selected by immunoscreening from the lambda gtll library were reacted against polyclonal anti-3-MC UDPGT serum to select monospecific anti UDPGT that recognize only 3-MC and BHA + 3-MC induced forms of UDPGT on a Western blot analysis of solubilized microsomes.

Lanes 1 and 2.	BHA + 3-MC microsome
Lanes 3 and 4.	Untreated microsome
Lanes 5 and 6.	BHA microsome
Lane 7.	Blank
Lanes 8 and 9.	3-MC microsome


1 2 3 4 5 6 7 8 9
MW
(K)
56K
54K

CHAPTER V

DISCUSSION

The biotransformation enzymes that seem to be differentially over-expressed in hostile chemical environments are collectively involved in the normal chemical defense arsenal of the cell. In this respect, UDPGT catalyzed glucuronidation of endogenous and exogenous chemicals has been acknowledged as the major pathway in biotransformation due to the ubiquitous nature and abundance of the co-substrate (UDPGA) in the cell. UDPGT is a bonafide microsomal membrane protein (Mackenzie, 1987) and, as such, the enzyme may have structural functions that have not been investigated. More than three forms of UDPGT isozymes have been demonstrated in mammalian livers, which have been distinguished based on induction, neonatal development, purification of specific enzyme (Dragacci et al., 1987) and substrate specificity (Mackenzie, 1987; Stewart and McCrary, 1987). Attempts in the past to chromatographically separate UDPGT from crude enzyme preparations have not been entirely successful. In this investigation, four isozymes of the enzyme were established based on ion-exchange and UDPGA-agarose affinity chromatographies. The isoforms are identified here as peaks A,B,C and D on the ion-exchange (Ananaba and Stewart, 1987). The peak A eluted without salt (KCl) addition, while peaks B

through D eluted with increasing salt concentration. Here, the differences in these proteins based on their affinity for the ion-exchange resin are apparent. In this regard, these proteins have been grouped as cationic (peak A) and anionic (peaks B through D). When these proteins were assayed for p-nitrophenol UDPGT activity, the cationic form had little or no measurable activity. However, on SDS/PAGE, two proteins of 52K and 54K were obtained from the cationic group. One of these proteins may be a post-translational modification form of the other, or two different proteins that co-eluted on UDPGA-agarose affinity columns. Most of the activity was detected in the anionic group. The absence of p-nitrophenol UDPGT activity in the cationic group in our hands, does not mean non-existence of other UDPGT forms in this region. Tephly et al. (1984) showed estrone-UDPGT activity and Falany et al. (1986) demonstrated beta-hydroxysteriod UDPGT in this region also. The distribution of p-nitrophenol UDPGT activity in this study may have been influenced by the purification conditions. Here, ammonium sulphate precipitation was omitted to cut down on the quantity of enzyme lost, as well as reduction in activity. Moreover, the purification buffer was always at a pH of 7.4, which can change the charge distribution on this enzyme since p-nitrophenol UDPGT has a pI of 6.8. Therefore, the isozymes that have been identified here

are different both in substrate specificity as well as charge and amino acid residues that make up the polypeptides.

To further purify these proteins, the anionic peaks were pooled and subjected to UDPGA-agarose affinity chromatography. The column was first eluted with the purification buffer, followed by 5 mM UDPGA elution. Two major peaks were obtained from the affinity purification. The fractions obtained here were assayed for p-nitrophenol UDPGT activity; measurable activity was only detected in the 5 mM UDPGA eluates. Any residual activity detected in the flow through fraction was interpreted as possible low affinity forms of the enzyme. The four isozymes that were identified here are consistent with the observations made earlier by Dr. McCrary (dissertation research). The purification procedure employed here was found to be very cost effective and less time consuming than those previously published. The distribution of the proteins was found not to be treatment dependent, rather xenobiotic dependent, since the untreated rat liver samples consistently did not have the peak B of the anionic group. We have putatively classified this peak as a xenobiotic specific form of the enzyme. Other interesting differences observed between the treated and untreated livers were the degree of liver

enlargement and lipid accumulation. Hepatomegaly and hyperlipidemia were not observed in the untreated samples. Among the treated samples, these symptoms were more pronounced in the BHA + 3-MC treatments, while there was no significant differences between the BHA and 3-MC treatments. Also, the treated animals showed appreciable change in body weight, which was additive in the BHA + 3-MC treatments compared to the BHA or 3-MC treatments. In comparison of the liver versus body weight ratios of the treated and untreated animals, a significant difference was observed ($p<.05$). While the difference between the BHA and 3-MC treatments was not significant, the combined treatment result was more than additive. Brown and Browne (1986) reported diffuse hyperplasia, hypertrophy, and fatty acid degeneration of fish hepatocytes in microscopically examined specimens. They reported enlarged liver when fish were treated with radioactive ash. It has been reported that pyrolysis of some organic materials (such as ash) can generate compounds like polycyclic aromatic hydrocarbons which can potentiate the expression of the genes for the biotransformation enzymes including UDPGT. These compounds may be responsible for the effects we as well as Brown and Browne have observed in our different systems.

When the affinity purified samples were subjected to SDS/PAGE analysis, we obtained two major proteins, M_r 80k and

57k. Bock et al. (1979); Tukey et al. (1981); Falany et al. (1983); Koster et al. (1986) have purified a 56k protein from rat liver, which they separately showed to have p-nitrophenol glucuronidation activity. By immunological and biochemical assays, we were able to demonstrate that the 57k protein is the active p-nitrophenol UDPGT. However, we were unable to assign a function to the 80k protein that copurified with the active protein in all the treatments. Nevertheless, this protein showed cross-reactivity with antibody raised against the 57k protein. The specific origin of this protein cannot be established until a function could be assigned to it. For now, we consider it a purification artifact that is unique to the system adopted here. However, UDPGT has been suggested to exist in precursor form which has a molecular weight greater than 57k (Iyanagi et al, 1986). It is then possible to postulate that the 80k protein obtained here may be the precursor UDPGT that is processed to the mature 57k. The affinity purified cationic group of proteins showed a 52k major band when analyzed on SDS/PAGE, with minor bands at about 60k, which we consider contaminants or low affinity forms of the enzyme from the anionic group. The 52k proteins have no measurable p-nitrophenol UDPGT activity.

In this work, total poly(A^+) RNA, polysomes and UDPGT specific polysomal poly(A^+) RNA were isolated. The differential accumulation of these molecules in different treatments confirmed the additive effect of the combined treatments that was observed when protein and enzyme activity were analyzed. Here, we demonstrated a stochiometric relationship between the protein (UDPGT), enzyme activity and transcriptional activity in treated animals. The polysome is a complex molecule containing among other protein translation factors, mostly ribosomes, mRNA, tRNA and nascent polypeptides. The over-accumulation of polysomes in treated tissues correlates with the accumulation of its individual components. Since rRNA and tRNA are transcribed, but not translated, we infer that the over-expression of these molecules and the differential expression of p-nitrophenol UDPGT observed in this investigation resulted from increased transcriptional events.

Jackson et al. (1986) and Mackenzie (1986) individually demonstrated that over-expression of mRNA in phenobarbital treated chicken embryo liver and rat liver mRNA, respectively, are transcriptionally mediated phenomena. Based on the structural unrelatedness of the inducers (BHA and 3-MC), we believe that they activate mRNA expression by different mechanisms. However, they seem to induce the expression of

p-nitrophenol UDPGT forms from different genes as indicated by the biochemical and immunological studies (Ananaba et al., 1987). Iversen et al. (1987) recently demonstrated synergy of phenobarbital and 3-methylcholanthrene in "superinduction" of cytochrome P-450C mRNA, but no corresponding increase in enzyme activity was observed. From these observations, it appears that synergy is a characteristic of some multiply induced systems. On the contrary, Stewart and McCrary (1987) showed that BHA + 3-MC induction does not result in additive expression of neither 1-naphthol nor phenolphathalein UDPGT. The differences in ability to exhibit synergy may be due to different mechanisms employed by the cell to activate the gene(s) that express these proteins.

To evaluate the immunological relationship among UDPGTs from different treatments, we immunoabsorbed out the activity from purified samples using rabbit anti-UDPGT made against the 3-MC induced enzyme. The differential inhibition obtained here was interpreted as an indication of different populations of anti-UDPGT in the serum. There are populations that recognize conserved sequences among UDPGT forms and those that recognize unique epitopes on different UDPGTs. This observation was substantiated by an antibody select study in which the affinity purified monospecific antisera recognized

only UDPGT from BHA + 3-MC and 3-MC in a Western blot analysis. The affinity purification process selected against BHA forms of p-nitrophenol UDPGT. Here, also, the intensity of the band was more pronounced in the BHA + 3-MC microsomal samples, which correlates with the synergistic expression of the specific mRNA and enzyme activity shown by Stewart and McCrary (1987). Furthermore, the cross-reactivity among different forms of UDPGT against antibodies from a particular treatment was determined by Ouchterlony double immunodiffusion and immunoblot assays.

UDPGT has been localized on endoplasmic reticulum membrane as well as the nuclear envelope. The activity of the enzyme on these membranes is stimulated by detergent treatments which affect the organization of the membranes (Mackenzie, 1987). We were able to show anti-UDPGT immune reactivity against unsolubilized microsomes. This is evidence that the enzyme is transmembrane and possibly located on the luminal side of the ER (Iyanagi et al., 1986). Iyanagi et al. (1986) have predicted the amino acid sequence of p-nitrophenol UDPGT from its cDNA, and showed that the hydrophobic domain (487-510) proximal to the -COOH terminus was surrounded on both sides by short segments of highly charged amino acids, which is characteristic of a halt-transfer signal that

functions to anchor the enzyme to the membrane, with -COOH terminal charged amino acids on the cytoplasmic surface.

In addition to the immunological studies which show the presence of conserved and variable epitopes among UDPGT forms, our tryptic digest analysis showed some homologous and unique amino acid sequences between p-nitrophenol UDPGT from BHA and 3-MC treated animals. Moreover, when the rat p-nitrophenol UDPGT was reacted against goat anti-mouse serum, cross-reactivity was observed. Based on these results, we infer that different species of UDPGT have a common origin, however, the functions of the conserved and unique regions in these enzymes have not been studied. Since UDP-glucuronic acid is a common donor substrate for all UDPGT, we postulate that at least some part of the conserved region corresponds to the binding site for the nucleotide and glucuronic acid moieties. Intra- and inter-species similarities in UDPGT forms have been well documented, however, little research has been done on the differences. The observation made here is consistent with the result of Iyanagi et al. (1986), who showed that nucleotides 35-371 are highly variable and nucleotides 372-1771 are highly conserved in p-nitrophenol and androsterone UDPGT. It is then possible to conclude that the differences in primary structure we have observed in

trypsin digest analysis are due to amino acids coded by the first four hundred nucleotides in the different UDPGT genes. A comparison of the hydropathy analysis of the p-nitrophenol UDPGT amino acid deduced by Iyanagi et al. (1986), and a phenobarbital inducible form of UDPGT deduced by Mackenzie (1986), show no major differences. Furthermore, Jackson et al. (1987) compared the hydrophobicity profiles of human (HLUG 25) and rat (RLUG 38) UDPGTs and found identical profiles. When they compared the cDNA sequences from these two species, they found 73% homology throughout the coding region, with two single-codon gaps in the human sequence required to maximize homology; however, homology between the 5'- and 3'-terminal non-coding sequences was not apparent. Recently, Harding et al. (1987) compared the amino acid sequence obtained from translation of the cDNA with the amino-terminal sequence of purified 17-beta-hydroxysteriod UDPGT protein and showed identical 38 amino acid sequences starting at residue 23 (end of cleaved signal sequence residues). Restriction map analysis performed for rat p-nitrophenol UDPGT cDNA by Iyanagi et al. (1986) and by Jackson et al. (1987) for a human UDPGT showed almost identical profiles. Jackson and his group showed that the human forms of UDPGT used UAA as the stop codon while rat forms used UAG for translation.

Several possible mechanisms have been postulated to explain the overexpression of biotransformation enzymes observed in xenobiotic induced tissues, particularly the liver, which is the most studied (Maniatis et al., 1987). Among these are the effects of cis-acting upstream and/or downstream sequences; sequences that influence gene expression irrespective of their orientation to the coding sequence (enhancers), the absence or presence of trans-acting factors in the cytosol that are activated by the inducers, and the possible existence of a separate gene for each enzyme form that is regulated by its own specific inducer(s). These genes are expressed synergistically or additively in combined xenobiotic stimulation.

Since we do not understand the exact regulatory mechanisms that control UDPGT expression, we can only speculate based on the studies that have been done on some closely related enzymes such as P-450 as well as other eukaryotic proteins. P-450 has been shown to belong to the same [Ah] battery of genes as UDPGT, and they are concomitantly expressed (Nebert and Gonzalez, 1987). Sogawa et al. (1986) showed that there are regulatory sequences on the P-450c promoter that are capable of conferring inducibility on a heterologous promoter independent of its orientation and distance from the subordinate promoter. These sequences were referred to as

"drug regulatory elements" (DRE), and they respond to 3-MC which is a prototype inducer for both P-450 and UDPGT. Work done by Hammer et al. (1987) on the diversity of alpha-fetoprotein gene expression showed that tissue specific trans-acting factors act on separate enhancers to bring about over-expression of the gene. Similar diversity has been observed in UDPGT gene expression, therefore, we cannot rule out the possibility of separate enhancers as a contributing factor to this phenomena. In addition, the role of trans-acting factors in eukaryotic gene expression has not been well studied, however, it has been demonstrated that co-operative binding of factors and accessibility of the binding sites may be required for the mechanism of gene activation (Sassone-Corsi and Borrelli, 1986). Moreover, the additive effects of tandemly reiterated regulatory elements on eukaryotic gene expression appear to be a general phenomenon (Maniatis et al., 1987). This has been demonstrated in heat-shock (Dudler and Travers, 1984) and beta-interferon genes (Goodbourn et al., 1985). There is also some evidence that the number of reiterated elements for efficient induction can vary among different cell types (Goodburn et al., 1985), which presumably reflects differences in the levels or kinds of trans-acting factors in these cells. One can then postulate that

both the differential expression and overexpression of UDPGT forms are controlled by reiterated regulatory elements. An alternative explanation of the differential expression of UDPGT genes involves tissue-specific enhancers which may contain different elements for binding constitutive and tissue specific factors, with binding of both types of factors required for full activation of the reiterated enhancers. It has been shown that some DNA segments act as enhancers only under specific conditions which are generated by the presence of an inducer. Inducers like BHA and 3-MC can act either directly or via a cellular mechanism for receiving and transducing the external signal, to effect the transcription of a specific gene or genes. Therefore, the inducers can be considered as types of trans-acting co-factors. Because of the limited knowledge presently available on eukaryotic gene expression, we cannot over-generalize the role of inducers, enhancers and trans-acting factors on gene expression.

We have demonstrated considerable amino acid differences between BHA and 3-MC forms of p-nitrophenol UDPGT, which implies the existence of more than one gene for this enzyme. We are cognizant of other factors that can result in over-expression of a single gene. For instance, the additive effect obtained here in the combined inductions, may be a

consequence of enhancer-like elements regulated by tissue-specific trans-acting factors activated by the inducers. The role of these putative sequences in modulating chemically induced gene expression has not be well investigated to contradict the existence of separate p-nitrophenol UDPGT genes induced by BHA and 3-MC. In addition, undermethylation has been documented as a possible mechanism in eukaryotic gene expression (Ploeg Vander et al., 1980). Actively transcribed genes are hypomethylated. Irrespective of the evidences in favor of methylation in gene expression, it is premature to speculate on the possibility of hypomethylation as a mechanism for the differential expression we have observed among the different treatments utilized in this study.

It should be apparent from this investigation that much of the molecular basis of chemical mediated over-expression of biotransformation enzymes is yet unsettled. However, the data obtained here have increased our knowledge of the relationships that exist among different forms of UDPGT, and advanced our understanding of the molecular basis of eukaryotic gene expression as well the evolution of these proteins.

CHAPTER VI

SUMMARY AND CONCLUSIONS

One of the many fundamental difficulties in eukaryotic molecular biology is to understand the mechanisms by which individual genes are expressed in response to exogenous and endogenous inducers. In this study, we have attempted to contribute to the general understanding of eukaryotic cell and molecular biology by elucidating some of the interrelationships that exist when 3-MC and/or BHA are used to activate UDPGT genes. The experimental results obtained in this investigation allow us to make several statements regarding the expression of rat liver p-nitrophenol UDPGT.

1. Although significant increase in macromolecule (e.g., proteins, lipids and nucleic acids) accumulation was observed in all the treatments compared to the untreated, hepatomegaly and hyperlipidemia were more prevalent in BHA + 3-MC treated animals compared to either BHA or 3-MC used alone.
2. Elimination of ammonium sulfate precipitation in the purification process and differences in amino acid residues among UDPGT isoforms resulted in an anionic distribution of p-nitrophenol UDPGT on ion-exchange chromatography.

3. Despite a bias towards 3-MC 57k protein, polyclonal antibody against 3-MC p-nitrophenol UDPGT crossreacts with UDPGT from the other treatments and a 3-MC 80k protein that co-purified with the 57k protein.
4. Goat anti-mouse UDPGT also cross-reacts with the 57k protein (p-nitrophenol UDPGT) from all the treatments, which demonstrates the inter and intra species conservation of amino acid sequences among these polypeptides.
5. Antibody against the 57k protein recognizes epitopes on unsolubilized microsomes, which indicates that this protein tranverses the microsomal membrane.
6. Differential inhibition of p-nitrophenol UDPGT activity by the polyclonal antibody indicates the uniqueness of the enzymes from different treatments. Moreover, the tryptic digest analysis as well as the antibody select studies validate the assumption that BHA and 3-MC activate the expression of p-nitrophenol UDPGT that are functionally similar, but structurally different.
7. The presence of numerous positive clones in the lambda gt11 cDNA library may reflect the multiplicity of UDPGT genes in rat liver and possible amplification of the positive clones.

8. Based on the information obtained here, we maintain that simultaneous exposure to multiple inducers results in over expression of some rat liver UDPGT genes, and conclude that the synergism we have observed may be due to the induction of separate UDPGT genes by BHA and 3-MC.

In this study, as in any other scientific investigation, we have answered some questions, and created new questions that require further study. Our lab is currently attempting to improve on the separation of the p-nitrophenol UDPGT isoforms, and at the same time scale up its yield for future in depth analysis. In addition, we are characterizing UDPGT cDNA inserts to establish specific nucleotide differences between BHA and 3-MC forms of the enzyme. To test some of the theoretical models presently available on the mechanism of genetic regulation, and the determination of UDPGT structure and forms, it will be essential to design and build chimeric molecules that have UDPGT coding sequences and known regulatory elements.

LITERATURE CITED

Abraham, R., K.F. Benitz, G. Patil and R. Lyon. 1986. Rapid induction of forestomach tumors in partially hepatectomized Wistar rats given butylated hydroxyanisole. Exp. Mol. Path. 44:14-20.

Amar-Costesec, A., H. Beaufay, M., Wibo, D. Thines-Sempoux, E. Feymas, M. Robbi and J. Berthet. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver II: Preparation and composition of the microsomal fraction. J. Cell Biol. 61:201-212.

Ananaba, G.A., J. Stewart, C. Boston and J. McCrary. 1987. Simultaneous 3-methylcholanthrene and antioxidant induction enhance the expression of rat liver p-nitrophenol UDP-glucuronyltransferase (Abstr.) Fed. Proc. 46:1415.

Ananaba, G.A. and J. Stewart. 1987. Effects of combined butylated hydroxyanisole and 3-methylcholanthrene treatments on the expression of p-nitrophenol UDP-glucuronyltransferase. (Abstr.) Georgia J. Sci. 45:11.

Antoine, B., J. Magdalou and G. Siest. 1984. Kinetic properties of UDP-glucuronyltransferase(s) in different membranes of rat liver cells. Xenobiotica 14:575-579.

Antoine, B., J. Magdalou and G. Siest. 1983. Functional heterogeneity of UDP-glucuronosyltransferase in different membranes of rat liver. Biochem. Pharmacol. 32:2629-2632.

Arias, I.M., L.M. Gartner, M. Cohen, J. Ben-Ezzer and A.J Levi. 1969. Chronic nonhemolytic unconjugated hyperbilirubineamia with glucuronyltransferase deficiency: Clincal, biochemical, pharmacologic, and genetic evidence for heterogeneity. Am. J. Med. 47:395-409.

Astrom, A., S. Maner and J.W. DePierre. 1986. Induction of cytochrome P-450 and related drug-metabolizing activities in the liver of different rodent species by 2-acetylaminofluorene or by 3-methylcholanthrene. Biochem. Pharmacol. 35:2703-2713.

Atkinson, P.A. and J.T. Lee. 1984. Co-translational excision of alpha-glucose and alpah-mannose in nascent vesicular stomatitis virus G protein. J. Cell Biol. 98:2245-2249.

Aviv, H. and P. Leder. 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. (USA). 69:1403-1412.

Barlow, A.K., J.E. Heckels and I.N. Clarke. 1987. Molecular cloning and expression of Neisseria Meningitidis Class 1 outer membrane protein in Escherichia coli K-12. Inf. Immun. 55:2734-2740.

Beaufay, H., A. Amar-Costesec, E. Feytmas, D. Thines-Sempoux, M. Wibo and Berthet, J. 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver I: Biochemical methods. J. Cell Biol. 61:188-200.

Belanger, P.M. 1985. Diurnal variations in the transferases and hydrolases involved in glucuronide and sulfate conjugation of rat liver. Drug Meta. Dis. 13:386-389.

Bochard, R.E., M.E. Welborn, N.B. Wiekhorst, D.W. Wilson and L.G. Hansen. 1975. Pharmacokinetics of polychlorinated biphenyl components in swine and sheep after a single oral dose. J. Pharm. Sci. 64:1294-1299.

Bock, K.W., D. Josting, N. Lilienblum and H. Pfiel. 1979. Purification of rat-liver microsomal UDP-glucuronyl-transferase: Two enyzme forms inducible by 3-methyl-cholanthrene or phenobarbital. Eur. J. Biochem. 98:19-26.

Bock, K.W., W. Lilienblum and H. Pfeil. 1982. Functional heterogeneity of UDP-glucuronsyltransferase activities in C57BL/6 and DBA/2 mice. Biochem. Pharm. 31:1273-1277.

Bock, K.W., W. Lilienblum, D. Ullrich and G. Fischer. 1984. Differential induction of UDP-glucuronosyltransferases and their 'permanent induction' in pre-neoplastic rat liver. Biochem. Soc. Trans. 12:55-58.

Bock, K.W., U.C. Von Clausbruch, R. Kaufman, W. Lilienblum, F. Oesch, H. Pfeil and K.L. Platt. 1980. Functional heterogeneity of UDP-glucuronyltransferase in rat tissue. Biochem. Pharmacol. 29:495-500.

Boutin, J.A., B. Antoine, A-M. Batt and G. Siest. 1984. Heterogeneity of hepatic microsomal UDP-glucuronsyl-transferase(s) activities: Comparison between human and mammalian species activities. Chem. Biol. Inter. 52:173-184.

Boutin, J.A., J. Thomassin, G. Siest and A. Carter. 1985. Heterogeneity of hepatic microsomal UDP-glucuronsyltransferase activities: conjugations of phenolic and monoterpenoid aglycones in control and induced rats and guinea pigs. Biochem. Pharmacol. 34:2235-2249.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Brown, I.V. and J.M. Browne. 1986. Carcinogenic potential of incinerated low-level radioactive waste: Cytopathologic evaluation. pp. 559-566. In: E.J. Burns, A.C. Upton, and G. Silini, (eds.). Radiation Carcinogenesis and DNA Alterations. Plenum Publishing Co. NY.

Burchell, B., M.R. Jackson, S.M.E. Kennedy, L.R. McCarthy and G.C. Barr. 1985. Characterization and regulation of hepatic UDP-glucuronyltransferase. In D.S. Davies, and A.R. Boobis, (eds). Microsomes and Drug Oxidation. Taylor and Francis, London.

Burchell, B., S. Kennedy, M. Jackson and L. McCarthy. 1984. The biosynthesis and induction of microsomal UDP-glucuronyltransferase in avian liver. Biochem. Soc. Trans. 12:50-53.

Burchell, B. and P. Weatherill. 1981. 4-nitrophenol UDP-glucuronyltransferase (rat liver). Methods in Enzymology. 77:17-50.

Castren, M. and A. Oikari. 1983. Optimal assay conditions for liver UDP-glucuronyltransferase from the rainbow trout, Salmo gairdneri. Comp. Biochem. Physiol. 76C:365-369.

Castuma, C.E. and R.R. Brenner. 1986. Cholesterol-dependent modification of microsomal dynamics and UDP-glucuronyltransferase kinetics. Biochem. 25:4733-4738.

Cha, Y-N and E. Bueding. 1979. Effect of 2(3)-tertbutyl-4-hydroxyanisole administration on the activities of several hepatic microsomal and cytoplasmic enzymes in mice. Biochem. Pharmacol. 28:1917-1921.

Cha, Y-N and H.S. Heine. 1982. Comparative effects of dietary administration of 2(3)-tert-butyl-4-hydroxy-anisole and 3,5-di-tert-butyl-4-hydroxytoluene on several hepatic enzyme activities in mice and rats. Cancer Res. 42:2609-2615.

Chen, C.N. and J. R. Andrade. 1976. Pharmacokinetic model for simultaneous determination of drug levels in organs and tissues. J. Pharm. Sci. 65:717-723.

Chirgwin, J.M., A.E. Przybylas, R.J. MacDonald and W.J. Rutter. 1979. Isolation of biologically active nucleic acid from sources enriched in ribonuclease. Biochem. 18:5294-5299.

Cleveland, D.W., S.G. Fischer, M.W. Kuschner and U.K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. 252:1102-1106.

Conney, A.H. 1967. Pharmacological implications of microsomal enzyme induction. Pharmacol. Rev. 19:317-320.

Conney, A.H., E.J. Pantuck, K.C. Hsiao, R. Kuntzman, A.P. Alvares and A. Kappas. 1977. Regulation of drug metabolism in man by environmental chemicals and diet. Fed. Proc. 36:1647-1652.

Coughtrie, N.W.H., B. Burchell, I.M. Shephard and J. R. Bend. 1987. Defective induction of phenol glucuronidation by 3-methylcholanthrene in Gunn rats is due to the absence of a specific UDP-glucuronosyltransferase isoenzyme. Mol. Pharmacol. 31:585-591.

Davis, L.G., M.D. Dibner, and J.F. Battey. 1986. Basic methods in molecular biology. Elsevier Publishing, New York.

Deliconstantines, G., M. Mykoniatis and D. Papodimitriou. 1986. Carbon tetrachloride modulates the rat hepatic microsomal UDP-glucuronyltransferase activity and membrane fluidity. Experienta. 42:181-183.

Dills, R.L. and C.D. Klaassen. 1986. The effect of inhibitors of mitochondrial energy production on hepatic glutathione, UDP-glucuronic acid, and adenosine 3'-phosphate-5'-phosphosulfate concentrations. Drug Meta. Dis. 14:190-196.

Dragacci, S., J. Thomassin, J. Maydalou, H. Souhaili El Amri, P. Boissel, and G. Siest. 1987. Properties of human hepatic UDP-glucuronosyltransferase relationship to other inducible enzymes in patients with cholestasis. Eur. J. Clin. Pharmacol. 32:485-491.

Dudler, R. and A. Travers. 1984. Upstream elements necessary for optimal function of the hsp 70 promoter in transformed flies. Cell 38:391-396.

Dutton, G.J. 1980. Glucuronidation of drugs and other compounds. CRC Press. Boca Raton, Florida.

_____. 1978. Developmental aspects of drug conjugation, with special reference to glucuronidation. Ann. Rev. Pharmacol. Toxicol. 18:17-35.

Eisenberg, M. and S.M. McLaughlin. 1976. Lipid bilayers as models for biological membranes. Biosciences. 26:436-442.

El-Dorry, H.A. 1986. Purification of mRNA coding for rat-liver fructose-1,6-bisphosphatase by polysome immunoabsorption. Biochim Biophys. Acta. 867:252-255.

Erickson, R.H., D. Zakim and D.A. Vessey. 1978. Preparation and properties of a phospholipid-free form of microsomal UDP-glucuronyltransferase. Biochem. 17:3706-3711.

Fagan, J.B., J.V. Pastewka, F.P. Guengerich and H.V. Gelboin 1983. Multiple cytochrome P-450s are translated from multiple messenger ribonucleic acids. Biochem. 22:1927-1934.

Falany, C.N., M.D. Green, E. Swain and T.R. Tephly. 1986. Substrate specificity and characterization of rat liver p-nitrophenol, 3 alpha-hydroxysteroid and 17 beta-hydroxysteroid UDP-glucuronyltransferase. Biochem. J. 238:65-73.

Falany, C.N. and T.R. Tephly. 1983. Separation, purification and characterization of three isoenzymes of UDP-glucuronyltransferase from rat liver microsomes. Arch. Biochem. Biophys. 227:248-258.

Finley, B.L., P.J. Ashley, A.G. Neptune and G.S. Yost. 1986. Substrate-selective induction of rabbit hepatic UDP-glucuronyltransferases by ethanol and other xenobiotics. Biochem. Pharmacol. 35:2875-2881.

Fischer, G. and A. Schauer. 1985. Increased UDP-glucuronyltransferase in putative preneoplastic foci of human liver after long-term use of oral contraceptives: Naturwissenschaften. 72:277-278.

Fouarge, M. 1986. Metabolism of 2-aminofluorene by rat small intestine fractions: Differential effect of intragastric versus intraperitoneal administration of 3-methylcholanthrene. Toxicol. Lett. 30:209-217.

French, T.B, H.M. Maul and G.G. Maul. 1986. Screening cDNA expression libraries with monoclonal and polyclonal antibodies using an amplified biotin-avidin peroxidase technique. Anal. Biochem. 156:417-423.

Gartner, L.M., K. Lee, S. Vaisman, D. Lane and I. Zarafu. 1977. Development of Bilirubin transport and metabolism in the newborn rhesus monkey. J. Pediatri. 90:513-531.

Giachelli, C.M. and C.J. Omiecinski. 1987. Developmental regulation of cytochrome P-450 genes in the rat. Mol. Pharmacol. 31:477-484.

Goodbourn, S., K. Zinn and T. Maniatis. 1985. Human beta-interferon gene expression is regulated by an inducible enhancer element. Cell 41:509-520.

Gorski, J.P. and C.B. Kasper. 1978. UDP-glucuronsyltransferase phospholipid dependence and properties of the reconstituted apoenzyme. Biochem. 17:4600-4605.

Green, M.D., C.N. Falany, R.B. Kirkpatrick and T.R. Tephly. 1985. Strain differences in purified rat hepatic 3 alpha-hydroxysteroid UDP-glucuronosyltransferase. Biochem. J. 230:403-409.

Grunstein, M. and D.S. Hogness. 1975. Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. 72:3961-3965.

Gubler, U. and B.J. Hoffman. 1983. A simple and efficient method for generating cDNA libraries. Gene. 25:263-269.

Hammer, R.E., R. Krumlauf, S.A. Camper, R.L. Brinster and S.M. Tilghman. 1987. Diversity of alpha-fetoprotein gene expression in mice is generated by a combination of separate enhance elements. Science 235:53-58.

Harding, D., S.M. Wilson, M.R. Jackson, B. Burchell, M.D. Green and T.R. Tephly. 1987. Nucleotide and deduced amino acid sequence of rat liver 17-beta hydroxysteriod UDP-glucuronyltransferase. Nucleic Acid Res. 15:3936.

Hartmann, F. and D.M. Bissell. 1982. Metabolism of heme and bilirubin in rat and human small intestinal mucosa. J. Clin. Invest. 70:23-29.

Hauser, S.C., J.C. Ziurys and J.L. Gollan. 1984. Subcellular distribution and regulation of hepatic bilirubin UDP-glucuronyltransferase. J. Biol. Chem. 259:4527-4533.

Hazelton, G.A., J.J. Hjelle and C.D. Klaassen. 1985. Effects of butylated hydroxyanisole on hepatic glucuronidation capacity of mice. Toxicol. Appl. Pharmacol. 78:280-290.

Hjelle, J.J. 1986. Hepatic UDP-glucuronic acid regulation during acetaminophen biotransformation in rats. J. Pharmacol. Expt. Therap. 237:750-756.

Hochman, Y. and D. Zakim. 1983. A comparison of the kinetic properties of two different forms of microsomal UDP-glucuronyltransferase. J. Biol. Chem. 258:4143-4146.

Hochman, Y., D. Zakim and D.A. Vessey. 1981. A kinetic mechanism for modulation of the activity of microsomal UDP-glucuronyltransferase by phospholipids: Effects of lyso-phosphatidylcholines. J. Biol. Chem. 256:4783-4788.

Hodgson, E. and F.E. Guthrie. (eds.) 1980. Introduction to biochemical toxicology. Blackwood Scientific Publication, Oxford, England.

Hucker, H.B. 1970. Species differences in drug metabolism. Ann. Rev. Pharmacol. 10:99-118.

Imaida, K., S. Fukushima, T. Shirai, N. Ohtani, K. Nakanishi and N. Ito. 1983. Promoting activities of butylated hydroxyanisole and butylated hydroxytoluene on two stage urinary bladder carcinogenesis and inhibition of glutamyl transpeptidase-positive foci development in the liver of rats. Carcinogenesis. 4:895-898.

Irshaid, Y.M. and T.R. Tephly. 1987. Isolation and purification of two human liver UDP-glucuronosyltransferases. Molecular Pharm. 31:27-34.

Isselbacher, K.J., M.F. Chrabas and R.C. Quinn. 1962. The solubilization and partial purification of a glucuronyltransferase from rabbit liver microsomes. J. Biol. Chem. 237:3033-3036.

Ito, F., S. Fukushima, K. Imaida, T. Sarata and T. Mauri. 1983. Induction of papilloma in the forestomach of hamsters by butylated hydroxyanisole. Gann. 74:459-461.

Iversen, P.L., L.I. Siegel, K. Rahner and E. Bresnick. 1987. Synergy of phenobarbital and 3-methylcholanthrene in "superinduction" of cytochrome P-450c mRNA but not enzyme activity. Biochem. Pharmacol. 36:3399-3403.

Iyanagi, T., M. Haniu, K. Sogawa, Y. Fujii-Kuriyama, S. Watanabe, J.E. Shively and K.F. Anan. 1986. Cloning and characterization of cDNA encoding 3-methylcholanthrene inducible rat mRNA for UDP-glucuronosyltransferase. J. Biol. Chem. 261:15609-15614.

Jackson, M.R., S.M.E. Kennedy, G. Lown and B. Burchell. 1986. Induction of UDP-glucuronyltransferase mRNA in embryonic chick livers by phenobarbital. Biochem. Pharmacol. 35:1191-1198.

Jackson, M.R., L.R. McCarthy, D. Harding, S. Wilson and M.W.H. Coughtrie and B. Burchell. 1987. Cloning of a human liver microsomal UDP-glucuronsyltransferase cDNA. Biochem. J. 242:581-588.

Jackson, M.R., L.R. McCarthy, R.B. Corser, G.C. Barr and B. Burchell. 1984. Cloning of cDNAs coding for rat hepatic microsomal UDP-glucuronyltransferases. Gene. 34:147-153.

Jacobson, M.M., W. Levin and A.H. Conney. 1975. Studies on bilirubin and steroid glucuronidation by rat liver microsomes. Biochem. Pharmacol. 24:655-662.

Koster, A.S.J., G. Schirmer and K.W. Bock. 1986. Immunological and functional characterization of UDP-glucuronosyltransferases. Biochem. Pharm. 35:3971-3975.

Kitchin, K.T. and M.T. Ebron. 1983. Maternal hepatic and embryonic effects of 1,2,4-trichlorobenzene in the rats. Environ. Res. 31:362-373.

Krajewska, W.M., W.N. Schmidt and L.S. Hnilica. 1986. In vitro isolation of rat liver and Novikoff hepatoma cytokeratin mRNAs. Mol. Cell. Biochem. 70:77-88.

Kraus, J.P. and L.E. Rosenberg. 1982. Purification of low-abundance messenger RNAs from rat liver by polysome immunoadsorption. Proc. Natl. Acad. Sci. 79:4015-5019.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T_4. Nature 227:680-685.

Leonard, T.B. and S.T. Jacob. 1977. Alterations in DNA-dependent RNA polymerase I and II from rat liver by thioacetamide: Preferential increase in the level of chromatin-associated nuclear RNA polymerase IB. Biochem. 16:4538-4544.

Lilienblum, W., A.K. Walli and K.W. Bock. 1982. Differential induction of rat liver microsomal UDP-glucuronosyltransferase activites by various inducing agents. Biochem. Pharmacol. 31:907-913.

Mackenzie, P. 1987. Personal communication.

Mackenzie, P.I. 1987. Rat liver UDP-glucuronosyltransferase: Identification of cDNAs encoding two enzymes which glucuronidate testosterone, dihydrotesterone, and Beta-estradiol. J. Biol. Chem. 262:9744-9749.

Mackenzie, P.I. 1986. Rat liver UDP-glucuronosyltransferase: Sequence and expression of a cDNA encoding a phenobarbital-inducible form. J. Biol. Chem. 261:6119-6125.

Mackenzie, P.I., F.J. Gonzalez and I.S. Owens. 1984a. Cloning and characterization of DNA complementary to rat liver UDP-glucuronsyltransferase mRNA. J. Biol. Chem. 259:12153-12160.

Mackenzie, P.I., L.M. Hjelmeland and I.S. Owens. 1984b. Purification and immunochemical characterization of a low-pI form of UDP-glucuronosyltransferase from mouse liver. Arch. Biochem. Biophys. 231:487-497.

Mackenzie, P.I., M.M Joffe, P.J. Munson and P.I. Owens. 1985. Separation of different UDP-glucuronosyltransferase activities according to charge heterogeneity by chromatofocusing using mouse liver microsomes: Three major types of aglycones. Biochem. Pharmacol. 34:737-746.

Magdalou, J., B. Antoine, D. Ratanasavanah and G. Siest. 1982. Membrane bound enzymes involved in drug metabolism. Enzyme. 28:41-47.

Maniatis, T., S. Goodbourn and J.A. Fischer. 1987. Regulation of inducible and tissue-specific gene expression. Science. 236:1237-1244.

Maniatis, T., E.F. Fritsch and J. Sambrook. 1982. Molecular cloning: A laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

McCrary, J.P. 1987. The selective induction of specific forms of UDP-glucuronyltransferase by antioxidants and 3-methylcholanthrene. Ph.D. Dissertation, Atlanta University.

Mellor, J.D. and R. Hobkirk. 1975. In vitro synthesis of estrogen glucuronides and sulfates by human renal tissue. Canadian J. Biochem. 53:779-783.

Miranda, C.L., R.L. Reed, P.R. Cheeke and D.R. Buhler. 1981. Protective effects of butylated hydroxyanisole against the acute toxicity of monocrotaline in mice. Toxicol. Appl. Pharm. 59:424-430.

Moldeus, P., L. Dock, Y-N. Cha, M. Berggen and B. Jerstrom. 1982. Elevation of conjugation capacity in isolated hepatocytes from BHA-treated mice. Biochem. Pharm. 31:1907-1910.

Nebert, D.W. and J.S. Felton. 1976. Importance of genetic factors influencing the metabolism of foreign compounds. Fed. Proc. 35:1133-1141.

Nebert, D.W. and F.J. Gonzalez. 1987. P-450 genes: Structure, evolution, and regulation. Ann. Rev. Biochem. 56:945-993.

Nelson, E. 1961. Kinetics of drug absorption, distribution, metabolism and excretion. J. Pharm. Sci. 50:191-198.

Norling, A. and O. Hanninen. 1974. Activation energy of glucuronide biosynthesis. Biochim. Biophys. Acta. 350: 183-192.

Ochs, D.C., E.H. McConkey and D.W. Sammons. 1981. Silver stains for proteins in polyacrylamide gels: A comparison of six methods. Electrophoresis. 2:304-307.

Okayama, H. and P. Berg. 1982. High efficiency cloning of full-length cDNA. Mol. Cell Biol. 2:161-170.

Ordman, A.B. and S. Kirkwood. 1977. UDP-glucose dehydrogenase kinetics and their mechanistic implications. Biochem. Biophys. Acta. 481:25-32.

Ouchterlony, O. 1968. Handbook of Immunodiffusion and Immunoelectrophoresis. Ann Arbor Science, Ann Arbor, MI.

Owens, I.S. and P.I. Mackenzie. 1984. Differential induction and glycosylation of UDP-glucuronosyltransferase. Biochem. Soc. Trans. 12:54-55.

Pelham, H.R.B. and R.J. Jackson. 1976. An efficient mRNA dependent translation system from reticulocyte lysates. Eur. J. Biochem. 67:247-256.

Peters, W.H.M. and P.L.M. Jansen. 1988. Immuno-characterization of UDP-glucuronyltransferase isoenzymes in human liver, intestine and kidney. Biochem. Pharmacol. 37:564-567.

Pickett, C.B., R.L. Jeter, J. Morin and A.Y.H. Lu. 1981. Electroimmunochemical quantitation of cytochrome P-450, cytochrome P-448, and epoxide hydrolase in rat liver microsome. J. Biol. Chem. 256:8815-8820.

Ploeg van der, L.H.T. and R.A. Flavell. 1980. DNA methylation in the human gamma-delta-globin locus in erythroid and nonerythroid tissues. Cell. 19:947-958.

Pogell, B.M. and L.F. Leloir. 1961. Nucleotide activation of liver microsomal glucuronidation. J. Biol. Chem. 236: 293-298.

Pucci, M.J., K.R. Jones, H.K. Kuramitsu and F. L. Macriona. 1987. Molecular cloning and characterization of the glucosyltransferase C gene (gtfc) from Streptococcus mutans LM7. Infect. Immu. 55:2176-2182.

Rance, M.J. and J.S. Shillingford. 1976. The role of the gut in the metabolism of strong analgesics. Biochem. Pharmacol. 25:735-741.

Roychowdhury, N., O. Portman, J. Roychowdhury, I.M. Arias and C.E. Cornelius. 1982. A primate model of Gilbert's syndrome. Gastroenterology. 82:1225.

Roychowdhury, Y., P.M. Novikoff, N. Roychowdhury and A.B. Novikoff. 1985. Distribution of UDP-glucuronosyl-transferase in rat tissue. Proc. Natl. Acad. Sci. 82:2990-2994.

Roychowdhury, J., N. Roychowdhury and I.M. Arias. 1984. UDP-glucuronosyltransferase deficiency in man and animals. Biochem. Soc. Trans. 12:81-83.

Saez, J.M., A.M. Morera, A. Dazard and J. Bertrand. 1972. Adrenal and testicular contribution to plasma oestrogens. J. Endo. 55:41-49.

Sakakibara, R., N. Tominaya, A. Sakai and M. Ishiguro. 1987. Electrophoretic separation of proteins on agarose gel-application for direct immunization with gel piece containing an antigen. Anal. Biochem. 162:150-155.

Salocks, C.B., D.P.H. Hsieh and J.L. Byard. 1981. Butylated hydroxytoluene pretreatment protects against cytotoxicity and reduces covalent binding of aflatoxin B_1 in primary hepatocyte cultures. Toxicol. Appl. Pharm. 59:331-345.

Sanvordeker, D.R. and H.J. Lambert. 1974. Environmental modification of mammalian drug metabolism and biological response. Drug Metabolism Rev. 3:201.

Sassone-Corsi, P. and E. Borrelli. 1986. Transcriptional regulation by trans-acting factors. TIG Aug:215-219.

Sato, K., A. Kitachara, Z. Yin, F. Waraga, K. Nishimura, I. Hatayama, T. Ebina, T. Yamazak, H. Tsuda and N. Ito. 1984. Induction by butylated hydroxyanisole of specific molecular forms of glutathione S-transferase and UDP-glucuronyltransferase and inhibition of development of gamma-glutamyl transpeptidase-positive foci in rat liver. Carcinogenesis. 5:473-477.

Schumaker, R., G.S. Rao, M.L. Rao and H. Breuer. 1972. Steroid glucuronyltransferases III: Oestradiol-17B 3-glucuronyltransferase of the mitochondria of pig intestine. Hoppe-Seyler's Z. Physiol. Chem. 353:1784-1788.

Schwenk, M. and M. Locher. 1985. 1-naphthol conjugation in isolated cells from liver, jejunum, ileum, colon and kidney of the guinea pig. Biochem. Pharm. 34:679-701.

Shipley, L.A. and M. Weiner. 1987. Effects of adenosine and uridine diphosphate glucuronic acid (UDPGA) synthesis in isolated rat hepatocytes. Biochem. Pharm. 36:2993-3000.

Singh, J. and L. R. Schwarz. 1981. Dependence of glucuronidation rate on UDP-glucuronic acid levels in isolated hepatocytes. Biochem. Pharmacol. 30:3252-3254.

Singh, O.M.P., A.B. Graham and G.C. Wood. 1981. The phospholipid-dependence of UDP-glucuronosyltransferase: Purification, delipidation and reconstitution of microsomal enzyme from guinea-pig liver. Eur. J. Biochem. 116:311-316.

Singh, S.V., D.D. Dao, C.A. Partridge, C. Theodore, S.K. Srivastava and Y.C. Awasthi. 1985. Different forms of human liver glutathione S-transferases arise from dimetric combinations of at least four immunologically and functionally distinct subunits. Biochem. J. 232: 781-790.

Smith, R.V. 1978. Metabolism of drugs and other foreign compounds by intestinal microorganisms. World Rev. Nutri. Diet. 29:60-68.

Sogawa, K., A. Fujisawa-Sehara, M. Yamane and Y. Fujii-Kuriyama. 1986. Location of regulatory elements responsible for drug induction in the rat cytochrome P-450c gene. Proc. Natl. Acad. Sci. 83:8044-8048.

Spater, H., N. Quintana, F. Becker and A.B. Novioff. 1983. Immunocytochemical localization of gamma-glutamyl-transferase induced hyperplastic nodules of rat liver. Proc. Natl. Acad. USA. 80:4742-4746.

Spindler, S., P. Siebert, F. Coffman and F. Frank. 1984. Isolation of biologically active mRNA. Biochem. Educ. 12:10-14.

Stewart, J. and C.M. Boston. 1987. Effect of pretreatment with 3-methylcholanthrene and several phenolic anti-oxidants on glutathione S-transferase activity in various tissues of female Sprague-Dawley rats. Xenobiotica 17:679-684.

Stewart, J. and J. McCrary. 1987. Comparison of the glucuronidation ability of liver microsomes from rats treated with 3-methylcholanthrene or phenolic anti-oxidants. Xenobiotica 17:1039-1046.

Sweeney, D.J. and L.A. Reinke. 1987. Effect of ethanol feeding on hepatic microsomal UDP-glucuronyltrans-ferase activity. Biochem. Pharmacol. 36:1381-1383.

Tukey, R.H., R. Robinson, B. Holm, C.N. Falany and T.R. Tephly. 1982. A procedure for the rapid separation and purification of UDP-glucuronosyltransferase from rabbit liver microsomes. Drug Meta. Disp. 10:97-101.

Tukey, R.H. and T.R. Tephly. 1981. Purification and properties of rabbit liver estrone and p-nitrophenol UDP-glucuronyltransferases. Arch. Biochem. Biophys. 209:565-578.

Vessey, D.A. and D. Zakim. 1972. Regulation of microsomal enzymes by phospholipids V: Kinetic studies of hepatic uridine diphosphate-glucuronyltransferase. J. Biol. Chem. 247:3023-3028.

Wang, L.H., D. Zakim, A.M. Rudolph and Z. Benet. 1986. Developmental alterations in hepatic UDP-glucuronosyltransferase: A comparison of the kinetic properties of enzymes from adult sheep and fetal lambs. Biochem. Pharmacol. 35:3065-3070.

Wattenberg, L.W. 1972. Inhibition of carcinogenic and toxic effects of polycyclic hydrocarbons by phenolic antioxidants and ethoxquin. J. Natl. Cancer Inst. 48:1425-1430.

Weber, K. and M. Osborne. 1968. The reliability of molecular weight determination by dodecyl-sulfate-polyacrylamide gel eletrophoresis. J. Biol. Chem. 244:4406-4412.

Whitmer, D.I., P.E. Russell, J.C. Ziurys and J. L. Gollan. 1985. Hepatic microsomal glucuronidation of bilirubin is modulated by the lipid microenvironment of membrane-bound substrate. J. Biol. Chem. 261:7170-7177.

Williams, G.M. 1984. Modulation of chemical carcinogenesis by xenobiotics. Fund. Appl. Toxicol. 4:325-344.

Williams, G.M., T. Tanaka and Y. Maeura. 1986. Dose-related inhibition of aflatoxin B_1 induced hepatocarcinogenesis by the phenolic antioxidants, butylated hydroxyanisole and butylated hydroxytoluene. Carcinogenesis. 1:1059-1063.

Wishart, G. 1978. Functional heterogeneity of UDP-glucuronsyltransferase as induced by its differential development and inducibility by glucocorticoids. Biochem. J. 174:485-489.

Wolkoff, A.W., J. Roychowdhury and I.M. Arias. 1983. The metabolic basis of inherited diseases. pp. 1385-1440, (J.B. Stanbury, J.B. Wyngaarden, D.S. Fredrickson, J.L. Goldstein and M.S. Brown (eds.). 5th ed. McGraw-Hill, NY.

Young, R.A. and R.W. Davis. 1983a. Efficient isolation of genes by using antibody probes. Proc. Natl. Acad. Sci. 80:1194-1198.

Young, R.A. and R.W. Davis. 1983b. Yeast RNA polymerase II genes: Isolation of genes using antibody probes. Science 222:778-782.

Yu, F-L. 1977. Mechanisms of aflatoxin B_1 inhibition of rat hepatic nuclear RNA synthesis. J. Biol. Chem. 252:32-45.

Yudelevich, A. 1973. Modification of leucine tRNA of Escherichia coli after bacteriophage T_4 infection. pp. 229-239. In: F.T. Kenney, B.A. Hamkalo, G. Favelukes and J.T. August (eds). Gene Expression and Its Regulation, Vol. 1, Plenum Press, New York.

Zakim, D. and D.A. Vessey. 1975. The effect of a temperature induced phase change within membrane lipids on the regulatory properties of microsomal uridine diphosphate glucuronyltransferase. J. Biol. Chem. 250:342-343.